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REPORTS

Axl1p sequence following Ser²⁰⁶ and occurs within the domain of Axl1p that shows homology with hDE (14). To delete the complete STE23 sequence and create the *ste23Δ::URA3* mutation, polymerase chain reaction (PCR) primers (5'-TCGGAAGACCTCAT-TCTTGCTCATTTTGATATTGCTC- TGTAGATTG-TACTGAGAGTGCAC-3'; and 5'-GCTACAAACAGC-GTCGACTTGAATGCCCGGACA TCTTCGACTGT-GCGGTATTTTACACCG-3') were used to amplify the URA3 sequence of pRS316, and the reaction product was transformed into yeast for one-step gene replacement [R. Rothstein, *Methods Enzymol.* 194, 281 (1991)]. To create the *axl1Δ::LEU2* mutation contained on p114, a 5.0-kb *Sal* I fragment from pAXL1 was cloned into pUC19, and an internal 4.0-kb *Hpa* I-*Xho* I fragment was replaced with a *LEU2* fragment. To construct the *ste23Δ::LEU2* allele (a deletion corresponding to 931 amino acids) carried on p153, a *LEU2* fragment was used to replace the 2.8-kb *Pml* I-Ecl136 II fragment of STE23, which occurs within a 6.2-kb *Hind* III-Bgl II genomic fragment carried on pSP72 (Promega). To create YEpMFA1, a 1.8-kb *Bam* HI fragment containing MFA1, from pKK16 [K. Kuchler, R. E. Sterne, J. Thormer, *EMBO J.* 8, 3973 (1989)], was ligated into the *Bam* HI site of YEp351 [J. E. Hill, A. M. Myers, T. J. Koerner, A. Tzagoloff, *Yeast* 2, 163 (1986)].

24. J. Chant and I. Herskowitz, *Cell* 65, 1203 (1991).
25. B. W. Matthews, *Acc. Chem. Res.* 21, 333 (1988).
26. K. Kuchler, H. G. Dohlman, J. Thormer, *J. Cell Biol.* 120, 1203 (1993); R. Kolling and C. P. Hollenberg, *EMBO J.* 13, 3261 (1994); C. Berkower, D. Loayza, S. Michaelis, *Mol. Biol. Cell* 5, 1185 (1994).
27. A. Bender and J. R. Pringle, *Proc. Natl. Acad. Sci. U.S.A.* 86, 9976 (1989); J. Chant, K. Corrado, J. R. Pringle, I. Herskowitz, *Cell* 65, 1213 (1991); S. Powers, E. Gonzales, T. Christensen, J. Cubert, D. Broek, *ibid.*, p. 1225; H. O. Park, J. Chant, I. Herskowitz, *Nature* 365, 269 (1993); J. Chant, *Trends Genet.* 10, 328 (1994); and J. R. Pringle, *J. Cell Biol.* 129, 751 (1995); J. Chant, M. Mischke, E. Mitchell, I. Herskowitz, J. R. Pringle, *ibid.*, p. 767.
28. G. F. Sprague Jr., *Methods. Enzymol.* 194, 77 (1991).
29. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
30. A W303 1A derivative, SY2625 (MATa *ura3-1 leu2-3 112 trp1-1 ade2-1 can1-100 sst1Δ mfa2Δ::FUS1-lacZ his3Δ::FUS1-HIS3*), was the parent strain for the mutant search. SY2625 derivatives for the mating assays, secreted pheromone assays, and the pulse-chase experiments included the following strains: Y49 (*ste22-1*), Y115 (*mfa1Δ::LEU2*), Y142 (*axl1::URA3*), Y173 (*axl1Δ::LEU2*), Y220 (*axl1::URA3 ste23Δ::URA3*), Y221 (*ste23Δ::URA3*), Y231 (*axl1Δ::LEU2 ste23Δ::LEU2*), and Y233 (*ste23Δ::LEU2*). MATa derivatives of SY2625 included the following strains: Y189 (SY2625 made MATa), Y278 (*ste22-1*), Y195 (*mfa1Δ::LEU2*), Y196 (*axl1Δ::LEU2*), and Y197 (*axl1::URA3*). The EG123 (MATa *leu2 ura3 trp1 can1 his4*) genetic background was used to create a set of strains for analysis of bud site selection. EG123 derivatives included the following strains: Y175 (*axl1Δ::LEU2*), Y223 (*axl1::URA3*), Y234 (*ste23Δ::LEU2*), and Y272 (*axl1Δ::LEU2 ste23Δ::LEU2*). MATa derivatives of EG123 included the following strains: Y214 (EG123 made MATa) and Y293 (*axl1Δ::LEU2*). All strains were generated by means of standard genetic or molecular methods involving the appropriate constructs (23). In particular, the *axl1 ste23* double mutant strains were created by crossing of the appropriate MATa *ste23* and MATa *axl1* mutants, followed by sporulation of the resultant diploid and isolation of the double mutant from nonparental di-tetrad tetrads. Gene disruptions were confirmed with either PCR or Southern (DNA) analysis.
31. p129 is a YEp352 [J. E. Hill, A. M. Myers, T. J. Koerner, A. Tzagoloff, *Yeast* 2, 163 (1986)] plasmid containing a 5.5-kb *Sal* I fragment of pAXL1. p151 was derived from p129 by insertion of a linker at the *Bgl* II site within AXL1, which led to an in-frame insertion of the hemagglutinin (HA) epitope (DQYYPDYA) (28) between amino acids 854 and 855 of the AXL1 prod-

uct. pC225 is a KS+ (Stratagene) plasmid containing a 0.5-kb *Bam* HI-*Sst* I fragment from pAXL1. Substitution mutations of the proposed active site of Axl1p were created with the use of pC225 and site-specific mutagenesis involving appropriate synthetic oligonucleotides (*axl1-H68A*, 5'-GTGCTCAGAAAGGCT-GCCAAACCGGC-3'; *axl1-E71A*, 5'-AAGAATCAT-GTGCGCACAAAGGTGCGC-3'; and *axl1-E71D*, 5'-AAGAATCATGTGATCAGAAAGGTGCGC-3'). The mutations were confirmed by sequence analysis. After mutagenesis, the 0.4-kb *Bam* HI-*Msc* I fragment from the mutagenized pC225 plasmids was transferred into pAXL1 to create a set of pRS316 plasmids carrying different AXL1 alleles, p124 (*axl1-H68A*), p130 (*axl1-E71A*), and p132 (*axl1-E71D*). Similarly, a set of HA-tagged alleles carried on YEp352 were created after replacement of the p151 *Bam* HI-*Msc* I fragment, to generate p161 (*axl1-E71A*), p162 (*axl1-*

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Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray

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A high-capacity system was developed to monitor the expression of many genes in parallel. Microarrays prepared by high-speed robotic printing of complementary DNAs on glass were used for quantitative expression measurements of the corresponding genes. Because of the small format and high density of the arrays, hybridization volumes of 2 microliters could be used that enabled detection of rare transcripts in probe mixtures derived from 2 micrograms of total cellular messenger RNA. Differential expression measurements of 45 *Arabidopsis* genes were made by means of simultaneous, two-color fluorescence hybridization.

The temporal, developmental, topographical, histological, and physiological patterns in which a gene is expressed provide clues to its biological role. The large and expanding database of complementary DNA (cDNA) sequences from many organisms (1) presents the opportunity of defining these patterns at the level of the whole genome.

For these studies, we used the small flowering plant *Arabidopsis thaliana* as a model organism. *Arabidopsis* possesses many advantages for gene expression analysis, including the fact that it has the smallest genome of any higher eukaryote examined to date (2). Forty-five cloned *Arabidopsis* cDNAs (Table 1), including 14 complete sequences and 31 expressed sequence tags (ESTs), were used as gene-specific targets. We obtained the ESTs by selecting cDNA clones at random from an *Arabidopsis* cDNA library. Sequence analysis revealed that 28 of the 31 ESTs matched sequences

in the database (Table 1). Three additional cDNAs from other organisms served as controls in the experiments.

The 48 cDNAs, averaging ~1.0 kb, were amplified with the polymerase chain reaction (PCR) and deposited into individual wells of a 96-well microtiter plate. Each sample was duplicated in two adjacent wells to allow the reproducibility of the arraying and hybridization process to be tested. Samples from the microtiter plate were printed onto glass microscope slides in an area measuring 3.5 mm by 5.5 mm with the use of a high-speed arraying machine (3). The arrays were processed by chemical and heat treatment to attach the DNA sequences to the glass surface and denature them (3). Three arrays, printed in a single lot, were used for the experiments here. A single microtiter plate of PCR products provides sufficient material to print at least 500 arrays.

Fluorescent probes were prepared from total *Arabidopsis* mRNA (4) by a single round of reverse transcription (5). The *Arabidopsis* mRNA was supplemented with human acetylcholine receptor (AChR) mRNA at a dilution of 1:10,000 (w/w) before cDNA synthesis, to provide an internal standard for calibration (5). The resulting fluorescently labeled cDNA mixture was hybridized to an array at high stringency (6) and scanned

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with a laser (3). A high-sensitivity scan gave signals that saturated the detector at nearly all of the *Arabidopsis* target sites (Fig. 1A). Calibration relative to the AChR mRNA standard (Fig. 1A) established a sensitivity limit of $\sim 1:50,000$. No detectable hybridization was observed to either the rat glucocorticoid receptor (Fig. 1A) or the yeast TRP4 (Fig. 1A) targets even at the highest scanning sensitivity. A moderate-sensitivity scan

of the same array allowed linear detection of the more abundant transcripts (Fig. 1B). Quantitation of both scans revealed a range of expression levels spanning three orders of magnitude for the 45 genes tested (Table 2). RNA blots (7) for several genes (Fig. 2) corroborated the expression levels measured with the microarray to within a factor of 5 (Table 2).

Differential gene expression was investi-

gated with a simultaneous, two-color hybridization scheme, which served to minimize experimental variation inherent in the comparison of independent hybridizations. Fluorescent probes were prepared from two mRNA sources with the use of reverse transcriptase in the presence of fluorescein- and lissamine-labeled nucleotide analogs, respectively (5). The two probes were then mixed together in equal proportions, hybridized to a single array, and scanned separately for fluorescein and lissamine emission after independent excitation of the two fluorophores (3).

To test whether overexpression of a single gene could be detected in a pool of total *Arabidopsis* mRNA, we used a microarray to analyze a transgenic line overexpressing the single transcription factor *HAT4* (8). Fluorescent probes representing mRNA from wild-type and *HAT4*-transgenic plants were labeled with fluorescein and lissamine, respectively; the two probes were then mixed and hybridized to a single array. An intense hybridization signal was observed at the position of the *HAT4* cDNA in the lissamine-specific scan (Fig. 1D), but not in the fluorescein-specific scan of the same array (Fig. 1C). Calibration with AChR mRNA added to the fluorescein and lissamine cDNA synthesis reactions at dilutions of 1:10,000 (Fig. 1C) and 1:100 (Fig. 1D), respectively, revealed a 50-fold elevation of *HAT4* mRNA in the transgenic line relative to its abundance in wild-type plants (Table 2). This magnitude of *HAT4* overexpression matched that inferred from the Northern (RNA) analysis within a factor of 2 (Fig. 2 and Table 2). Expression of all the other genes monitored on the array differed by less than a factor of 5 between *HAT4*-transgenic and wild-type plants (Fig. 1, C

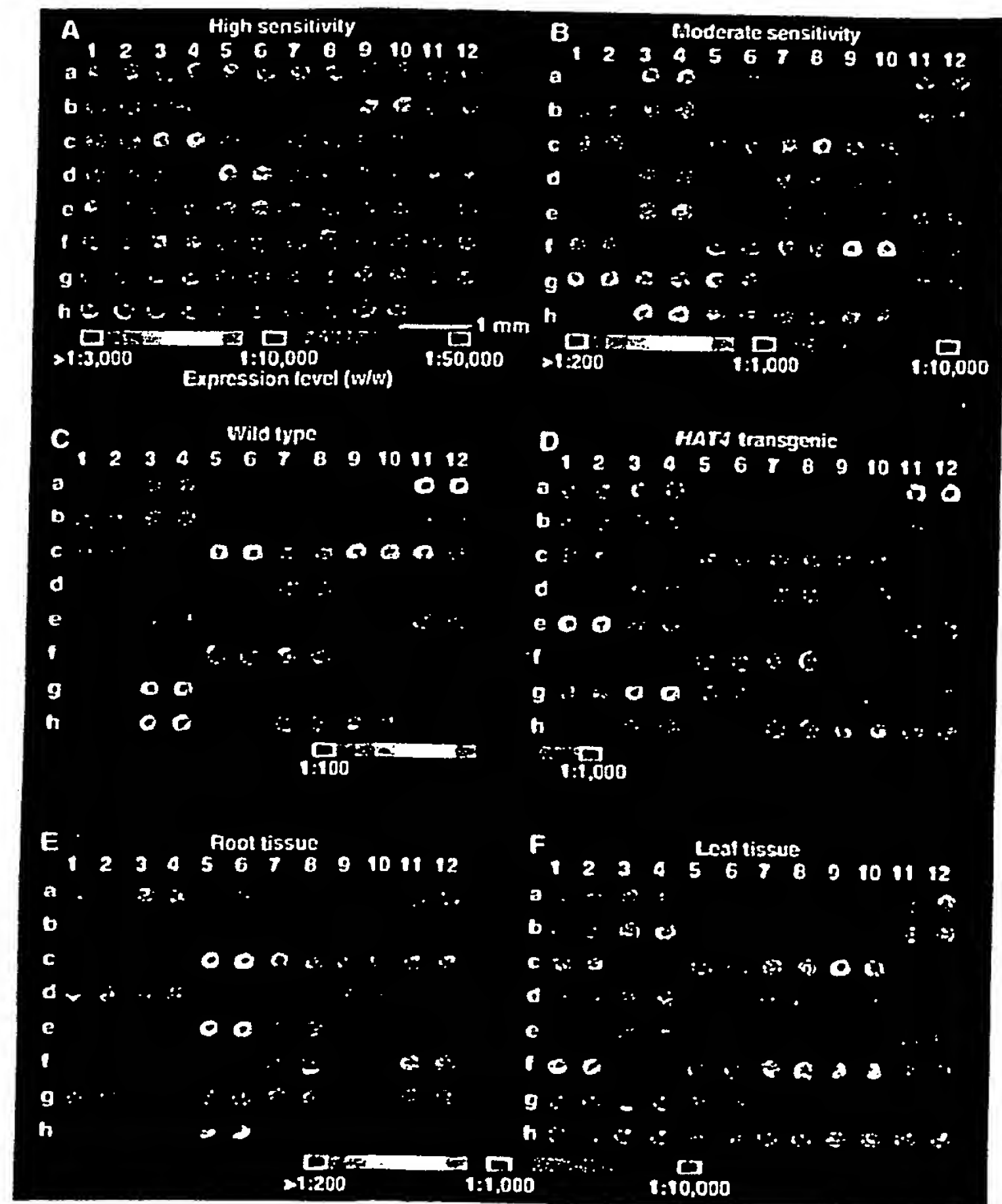


Fig. 1. Gene expression monitored with the use of cDNA microarrays. Fluorescent scans represented in pseudocolor correspond to hybridization intensities. Color bars were calibrated from the signal obtained with the use of known concentrations of human AChR mRNA in independent experiments. Numbers and letters on the axes mark the position of each cDNA. (A) High-sensitivity fluorescein scan after hybridization with fluorescein-labeled cDNA derived from wild-type plants. (B) Same array as in (A) but scanned at moderate sensitivity. (C and D) A single array was probed with a 1:1 mixture of fluorescein-labeled cDNA from wild-type plants and lissamine-labeled cDNA from *HAT4*-transgenic plants. The single array was then scanned successively to detect the fluorescein fluorescence corresponding to mRNA from wild-type plants (C) and the lissamine fluorescence corresponding to mRNA from *HAT4*-transgenic plants (D). (E and F) A single array was probed with a 1:1 mixture of fluorescein-labeled cDNA from root tissue and lissamine-labeled cDNA from leaf tissue. The single array was then scanned successively to detect the fluorescein fluorescence corresponding to mRNAs expressed in roots (E) and the lissamine fluorescence corresponding to mRNAs expressed in leaves (F).

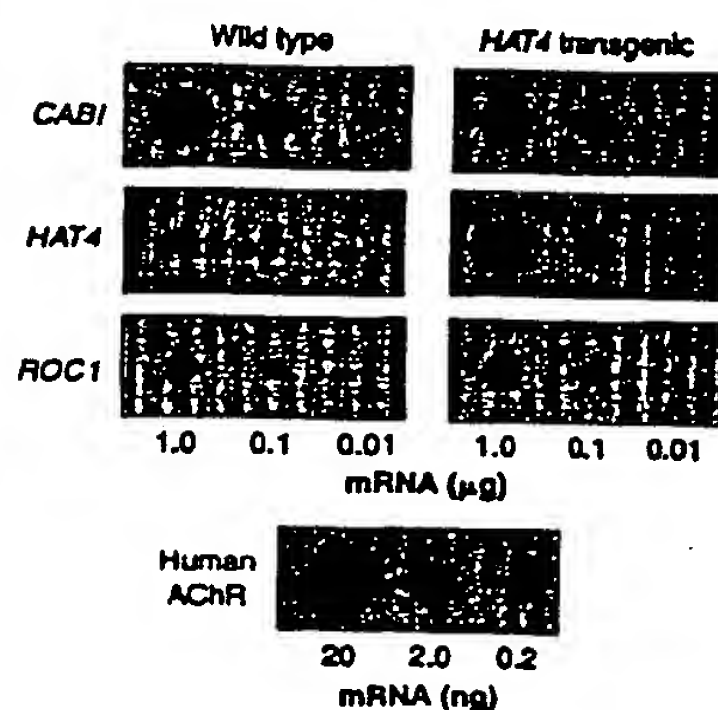


Fig. 2. Gene expression monitored with RNA (Northern) blot analysis. Designated amounts of mRNA from wild-type and *HAT4*-transgenic plants were spotted onto nylon membranes and probed with the cDNAs indicated. Purified human AChR mRNA was used for calibration.

and D, and Table 2). Hybridization of fluorescein-labeled glucocorticoid receptor cDNA (Fig. 1C) and lissamine-labeled TRP4 cDNA (Fig. 1D) verified the presence of the negative control targets and the lack of optical cross talk between the two fluorophores.

To explore a more complex alteration in expression patterns, we performed a second two-color hybridization experiment with fluorescein- and lissamine-labeled probes prepared from root and leaf mRNA, respectively. The scanning sensitivities for the two fluorophores were normalized by matching the signals resulting from AChR

mRNA, which was added to both cDNA synthesis reactions at a dilution of 1:1000 (Fig. 1, E and F). A comparison of the scans revealed widespread differences in gene expression between root and leaf tissue (Fig. 1, E and F). The mRNA from the light-regulated *CAB1* gene was ~500-fold more abundant in leaf (Fig. 1F) than in root tissue (Fig. 1E). The expression of 26 other genes differed between root and leaf tissue by more than a factor of 5 (Fig. 1, E and F).

The HAT4-transgenic line we examined has elongated hypocotyls, early flowering, poor germination, and altered pigmentation (8). Although changes in expression were

observed for HAT4, large changes in expression were not observed for any of the other 44 genes we examined. This was somewhat surprising, particularly because comparative analysis of leaf and root tissue identified 27 differentially expressed genes. Analysis of an expanded set of genes may be required to identify genes whose expression changes upon HAT4 overexpression; alternatively, a comparison of mRNA populations from specific tissues of wild-type and HAT4-transgenic plants may allow identification of downstream genes.

At the current density of robotic printing, it is feasible to scale up the fabrication process to produce arrays containing 20,000 cDNA targets. At this density, a single array would be sufficient to provide gene-specific targets encompassing nearly the entire repertoire of expressed genes in the *Arabidopsis* genome (2). The availability of 20,274 ESTs from *Arabidopsis* (1, 9) would provide a rich source of templates for such studies.

The estimated 100,000 genes in the human genome (10) exceeds the number of *Arabidopsis* genes by a factor of 5 (2). This modest increase in complexity suggests that similar cDNA microarrays, prepared from the rapidly growing repertoire of human ESTs (1), could be used to determine the expression patterns of tens of thousands of human genes in diverse cell types. Coupling an amplification strategy to the reverse transcription reaction (11) could make it feasible to monitor expression even in minute tissue samples. A wide variety of acute and chronic physiological and pathological conditions might lead to characteristic changes in the patterns of gene expression in peripheral blood cells or other easily sampled tissues. In concert with cDNA microarrays for monitoring complex expression patterns, these tissues might therefore serve as sensitive *in vivo* sensors for clinical diagnosis. Microarrays of cDNAs could thus provide a useful link between human gene sequences and clinical medicine.

Table 2. Gene expression monitoring by microarray and RNA blot analyses; tg, HAT4-transgenic. See Table 1 for additional gene information. Expression levels (w/w) were calibrated with the use of known amounts of human AChR mRNA. Values for the microarray were determined from microarray scans (Fig. 1); values for the RNA blot were determined from RNA blots (Fig. 2).

| Gene | Expression level (w/w) | |
|------------------|------------------------|----------|
| | Microarray | RNA blot |
| <i>CAB1</i> | 1:48 | 1:83 |
| <i>CAB1</i> (tg) | 1:120 | 1:150 |
| <i>HAT4</i> | 1:8300 | 1:6300 |
| <i>HAT4</i> (tg) | 1:150 | 1:210 |
| <i>ROC1</i> | 1:1200 | 1:1800 |
| <i>ROC1</i> (tg) | 1:260 | 1:1300 |

Table 1. Sequences contained on the cDNA microarray. Shown is the position, the known or putative function, and the accession number of each cDNA in the microarray (Fig. 1). All but three of the ESTs used in this study matched a sequence in the database. NADH, reduced form of nicotinamide adenine dinucleotide; ATPase, adenosine triphosphatase; GTP, guanosine triphosphate.

| Position | cDNA | Function | Accession number |
|----------|--------------|-------------------------------|------------------|
| a1, 2 | AChR | Human AChR | - |
| a3, 4 | EST3 | Actin | H36236 |
| a5, 6 | EST6 | NADH dehydrogenase | Z27010 |
| a7, 8 | AAC1 | Actin 1 | M20016 |
| a9, 10 | EST12 | Unknown | U36594† |
| a11, 12 | EST13 | Actin | T45783 |
| b1, 2 | <i>CAB1</i> | Chlorophyll a/b binding | M85150 |
| b3, 4 | EST17 | Phosphoglycerate kinase | T44490 |
| b5, 6 | GA4 | Gibberellic acid biosynthesis | L37126 |
| b7, 8 | EST19 | Unknown | U36595† |
| b9, 10 | <i>GBF-1</i> | G-box binding factor 1 | X63894 |
| b11, 12 | EST23 | Elongation factor | X52256 |
| c1, 2 | EST29 | Aldolase | T04477 |
| c3, 4 | <i>GBF-2</i> | G-box binding factor 2 | X63895 |
| c5, 6 | EST34 | Chloroplast protease | R87034 |
| c7, 8 | EST35 | Unknown | T14152 |
| c9, 10 | EST41 | Catalase | T22720 |
| c11, 12 | rGR | Rat glucocorticoid receptor | M14053 |
| d1, 2 | EST42 | Unknown | U36596† |
| d3, 4 | EST45 | ATPase | J04185 |
| d5, 6 | <i>HAT1</i> | Homeobox-leucine zipper 1 | U09332 |
| d7, 8 | EST46 | Light harvesting complex | T04063 |
| d9, 10 | EST49 | Unknown | T76267 |
| d11, 12 | <i>HAT2</i> | Homeobox-leucine zipper 2 | U09335 |
| e1, 2 | <i>HAT4</i> | Homeobox-leucine zipper 4 | M90394 |
| e3, 4 | EST50 | Phosphoribulokinase | T04344 |
| e5, 6 | <i>HAT5</i> | Homeobox-leucine zipper 5 | M90416 |
| e7, 8 | EST51 | Unknown | Z33675 |
| e9, 10 | <i>HAT22</i> | Homeobox-leucine zipper 22 | U09336 |
| e11, 12 | EST52 | Oxygen evolving | T21749 |
| f1, 2 | EST59 | Unknown | Z34607 |
| f3, 4 | <i>KNAT1</i> | Knotted-like homeobox 1 | U14174 |
| f5, 6 | EST60 | RuBisCO small subunit | X14564 |
| f7, 8 | EST69 | Translation elongation factor | T42799 |
| f9, 10 | <i>PPH1</i> | Protein phosphatase 1 | U34803 |
| f11, 12 | EST70 | Unknown | T44621 |
| g1, 2 | EST75 | Chloroplast protease | T43698 |
| g3, 4 | EST78 | Unknown | R65481 |
| g5, 6 | <i>ROC1</i> | Cyclophilin | L14844 |
| g7, 8 | EST82 | GTP binding | X59152 |
| g9, 10 | EST83 | Unknown | Z33795 |
| g11, 12 | EST84 | Unknown | T45278 |
| h1, 2 | EST91 | Unknown | T13832 |
| h3, 4 | EST96 | Unknown | R64816 |
| h5, 6 | <i>SAR1</i> | Synaptobrevin | M90418 |
| h7, 8 | EST100 | Light harvesting complex | Z18205 |
| h9, 10 | EST103 | Light harvesting complex | X03909 |
| h11, 12 | <i>TRP4</i> | Yeast tryptophan biosynthesis | X04273 |

*Proprietary sequence of Stratagene (La Jolla, California).

†No match in the database; novel EST.

REFERENCES AND NOTES

1. The current EST database (dbEST release 091495) from the National Center for Biotechnology Information (Bethesda, MD) contains a total of 322,225 entries, including 255,645 from the human genome and 21,044 from Arabidopsis. Access is available via the World Wide Web (<http://www.ncbi.nlm.nih.gov>).
2. E. M. Meyerowitz and R. E. Pruitt, *Science* 229, 1214 (1985); R. E. Pruitt and E. M. Meyerowitz, *J. Mol. Biol.* 187, 169 (1986); I. Hwang et al., *Plant J.* 1, 367 (1991); P. Jarvis et al., *Plant Mol. Biol.* 24, 685 (1994); L. Le Guen et al., *Mol. Gen. Genet.* 245, 390 (1994).
3. D. Shalon, thesis, Stanford University (1995); and P. O. Brown, in preparation. Microarrays were fabricated on poly-L-lysine-coated microscope slides (Sigma) with a custom-built arraying machine fitted with one printing tip. The tip loaded 1 μ l of PCR product (0.5 mg/ml) from 96-well microtiter plates and deposited \sim 0.005 μ l per slide on 40 slides at a spacing of 500 μ m. The printed slides were rehydrated for 2 hours in a humid chamber, snap-dried at 100°C for 1 min, rinsed in 0.1% SDS, and treated with 0.05% succinic anhydride prepared in buffer consisting of 50% 1-methyl-2-pyrrolidinone and 50% boric acid. The cDNA on the slides was denatured in distilled water for 2 min at 90°C immediately before use. Microarrays were scanned with a laser fluorescent scanner that contained a computer-controlled XY stage and a microscope objective. A mixed gas, multiline laser allowed sequential excitation of the two fluorophores. Emitted light was split according to wavelength and detected with two photomultiplier tubes. Signals were read into a PC with the use of a 12-bit analog-to-digital board. Additional details of microarray fabrication and use may be obtained by means of e-mail (pbrown@cimgm.stanford.edu).
4. F. M. Ausubel et al., Eds., *Current Protocols in Molecular Biology* (Greene & Wiley Interscience, New York, 1994), pp. 4.3.1–4.3.4.
5. Polyadenylated [poly(A)]⁺ mRNA was prepared from total RNA with the use of Oligotex-dT resin (Qiagen). Reverse transcription (RT) reactions were carried out with a StrataScript RT-PCR kit (Stratagene) modified as follows: 50- μ l reactions contained 0.1 μ g/ μ l of Arabidopsis mRNA, 0.1 ng/ μ l of human AChR mRNA, 0.05 μ g/ μ l of oligo(dT) (21-mer), 1 \times first strand buffer, 0.03 U/ μ l of ribonuclease block, 500 μ M deoxyadenosine triphosphate (dATP), 500 μ M deoxyguanosine triphosphate, 500 μ M dTTP, 40 μ M deoxycytosine triphosphate (dCTP), 40 μ M fluorescein-12-dCTP (or Issamine-5-dCTP), and 0.03 U/ μ l of StrataScript reverse transcriptase. Reactions were incubated for 60 min at 37°C, precipitated with ethanol, and resuspended in 10 μ l of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Samples were then heated for 3 min at 94°C and chilled on ice. The RNA was degraded by adding 0.25 μ l of 10 N NaOH followed by a 10-min incubation at 37°C. The samples were neutralized by addition of 2.5 μ l of 1 M Tris-HCl (pH 8.0) and 0.25 μ l of 10 N HCl and precipitated with ethanol. Pellets were washed with 70% ethanol, dried to completion in a speedvac, resuspended in 10 μ l of H₂O, and reduced to 3.0 μ l in a speedvac. Fluorescent nucleotide analogs were obtained from New England Nuclear (DuPont).
6. Hybridization reactions contained 1.0 μ l of fluorescent cDNA synthesis product (5) and 1.0 μ l of hybridization buffer (10 \times saline sodium citrate (SSC) and 0.2% SDS). The 2.0- μ l probe mixtures were aliquoted onto the microarray surface and covered with cover slips (12 mm round). Arrays were transferred to a hybridization chamber (3) and incubated for 18 hours at 65°C. Arrays were washed for 5 min at room temperature (25°C) in low-stringency wash buffer (1 \times SSC and 0.1% SDS), then for 10 min at room temperature in high-stringency wash buffer (0.1 \times SSC and 0.1% SDS). Arrays were scanned in 0.1 \times SSC with the use of a fluorescence laser-scanning device (3).
7. Samples of poly(A)⁺ mRNA (4, 5) were spotted onto nylon membranes (Nytan) and crosslinked with ultraviolet light with the use of a Stratalinker 1800 (Stratagene). Probes were prepared by random priming with the use of a Prime-It II kit (Stratagene) in the presence of [³²P]dATP. Hybridizations were carried out according to the instructions of the manufacturer. Quantitation was performed on a PhosphorImager (Molecular Dynamics).
8. M. Schena and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* 89, 3894 (1992); M. Schena, A. M. Lloyd, R. W. Davis, *Genes Dev.* 7, 367 (1993); M. Schena and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* 91, 8393 (1994).
9. H. Hofte et al., *Plant J.* 4, 1051 (1993); T. Newman et al., *Plant Physiol.* 106, 1241 (1994).
10. N. E. Morton, *Proc. Natl. Acad. Sci. U.S.A.* 88, 7474 (1991); E. D. Green and R. H. Waterston, *J. Am. Med. Assoc.* 266, 1966 (1991); C. Bellenne-Chantelot, *Cell* 70, 1059 (1992); D. R. Cox et al., *Science* 265, 2031 (1994).
11. E. S. Kawasaki et al., *Proc. Natl. Acad. Sci. U.S.A.* 85, 5698 (1988).
12. The laser fluorescent scanner was designed and fabricated in collaboration with S. Smith of Stanford University. Scanner and analysis software was developed by R. X. Xia. The succinic anhydride reaction was suggested by J. Mulligan and J. Van Ness of Darwin Molecular Corporation. Thanks to S. Theologis, C. Somerville, K. Yamamoto, and members of the laboratories of R.W.D. and P.O.B. for critical comments. Supported by the Howard Hughes Medical Institute and by grants from NIH (R21HG00450) (P.O.B.) and R37AG00198 (R.W.D.) and from NSF (MCB9106011) (R.W.D.) and by an NSF graduate fellowship (D.S.). P.O.B. is an assistant investigator of the Howard Hughes Medical Institute.

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Gene Therapy in Peripheral Blood Lymphocytes and Bone Marrow for ADA⁻ Immunodeficient Patients

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Adenosine deaminase (ADA) deficiency results in severe combined immunodeficiency, the first genetic disorder treated by gene therapy. Two different retroviral vectors were used to transfer ex vivo the human ADA minigene into bone marrow cells and peripheral blood lymphocytes from two patients undergoing exogenous enzyme replacement therapy. After 2 years of treatment, long-term survival of T and B lymphocytes, marrow cells, and granulocytes expressing the transferred ADA gene was demonstrated and resulted in normalization of the immune repertoire and restoration of cellular and humoral immunity. After discontinuation of treatment, T lymphocytes, derived from transduced peripheral blood lymphocytes, were progressively replaced by marrow-derived T cells in both patients. These results indicate successful gene transfer into long-lasting progenitor cells, producing a functional multilineage progeny.

Severe combined immunodeficiency associated with inherited deficiency of ADA (1) is usually fatal unless affected children are kept in protective isolation or the immune system is reconstituted by bone marrow transplantation from a human leukocyte antigen (HLA)-identical sibling donor (2). This is the therapy of choice, although it is available only for a minority of patients. In recent years, other forms of therapy have been developed, including transplants from haploidentical donors (3, 4), exogenous enzyme replacement (5), and somatic-cell gene therapy (6–9).

We previously reported a preclinical model in which ADA gene transfer and expression

successfully restored immune functions in human ADA-deficient (ADA⁻) peripheral blood lymphocytes (PBLs) in immunodeficient mice in vivo (10, 11). On the basis of these preclinical results, the clinical application of gene therapy for the treatment of ADA⁻ SCID (severe combined immunodeficiency disease) patients who previously failed exogenous enzyme replacement therapy was approved by our Institutional Ethical Committees and by the Italian National Committee for Bioethics (12). In addition to evaluating the safety and efficacy of the gene therapy procedure, the aim of the study was to define the relative role of PBLs and hematopoietic stem cells in the long-term reconstitution of immune functions after retroviral vector-mediated ADA gene transfer. For this purpose, two structurally identical vectors expressing the human ADA complementary DNA (cDNA), distinguishable by the presence of alternative restriction sites in a nonfunctional region of the viral long-terminal repeat (LTR), were used to transduce PBLs and bone marrow (BM) cells independently. This procedure allowed identification of the origin of

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Differential gene expression in drug metabolism and toxicology: practicalities, problems and potential

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1. An important feature of the work of many molecular biologists is identifying which genes are switched on and off in a cell under different environmental conditions or subsequent to xenobiotic challenge. Such information has many uses, including the deciphering of molecular pathways and facilitating the development of new experimental and diagnostic procedures. However, the student of gene hunting should be forgiven for perhaps becoming confused by the mountain of information available as there appears to be almost as many methods of discovering differentially expressed genes as there are research groups using the technique.

2. The aim of this review was to clarify the main methods of differential gene expression analysis and the mechanistic principles underlying them. Also included is a discussion on some of the practical aspects of using this technique. Emphasis is placed on the so-called 'open' systems, which require no prior knowledge of the genes contained within the study model. Whilst these will eventually be replaced by 'closed' systems in the study of human, mouse and other commonly studied laboratory animals, they will remain a powerful tool for those examining less fashionable models.

3. The use of suppression-PCR subtractive hybridization is exemplified in the identification of up- and down-regulated genes in rat liver following exposure to phenobarbital, a well-known inducer of the drug metabolizing enzymes.

4. Differential gene display provides a coherent platform for building libraries and microchip arrays of 'gene fingerprints' characteristic of known enzyme inducers and xenobiotic toxicants, which may be interrogated subsequently for the identification and characterization of xenobiotics of unknown biological properties.

Introduction

It is now apparent that the development of almost all cancers and many non-neoplastic diseases are accompanied by altered gene expression in the affected cells compared to their normal state (Hunter 1991, Wynford-Thomas 1991, Vogelstein and Kinzler 1993, Semenza 1994, Cassidy 1995, Kleinjan and Van Hegningen 1998). Such changes also occur in response to external stimuli such as pathogenic micro-organisms (Rohn *et al.* 1996, Singh *et al.* 1997, Griffin and Krishna 1998, Lunney 1998) and xenobiotics (Sewall *et al.* 1995, Dogra *et al.* 1998, Ramana and Kohli 1998), as well as during the development of undifferentiated cells (Hecht 1998, Rudin and Thompson 1998, Schneider-Maunoury *et al.* 1998). The potential medical and therapeutic benefits of understanding the molecular changes which occur in any given cell in progressing from the normal to the 'altered' state are enormous. Such profiling essentially provides a 'fingerprint' of each step of a

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cell's development or response and should help in the elucidation of specific and sensitive biomarkers representing, for example, different types of cancer or previous exposure to certain classes of chemicals that are enzyme inducers.

In drug metabolism, many of the xenobiotic-metabolizing enzymes (including the well-characterized isoforms of cytochrome P450) are inducible by drugs and chemicals in man (Pelkonen *et al.* 1998), predominantly involving transcriptional activation of not only the cognate cytochrome P450 genes, but additional cellular proteins which may be crucial to the phenomenon of induction. Accordingly, the development of methodology to identify and assess the full complement of genes that are either up- or down-regulated by inducers are crucial in the development of knowledge to understand the precise molecular mechanisms of enzyme induction and how this relates to drug action. Similarly, in the field of chemical-induced toxicity, it is now becoming increasingly obvious that most adverse reactions to drugs and chemicals are the result of multiple gene regulation, some of which are causal and some of which are casually-related to the toxicological phenomenon *per se*. This observation has led to an upsurge in interest in gene-profiling technologies which differentiate between the control and toxin-treated gene pools in target tissues and is, therefore, of value in rationalizing the molecular mechanisms of xenobiotic-induced toxicity. Knowledge of toxin-dependent gene regulation in target tissues is not solely an academic pursuit as much interest has been generated in the pharmaceutical industry to harness this technology in the early identification of toxic drug candidates, thereby shortening the developmental process and contributing substantially to the safety assessment of new drugs. For example, if the gene profile in response to say a testicular toxin that has been well-characterized *in vivo* could be determined in the testis, then this profile would be representative of all new drug candidates which act via this specific molecular mechanism of toxicity, thereby providing a useful and coherent approach to the early detection of such toxicants. Whereas it would be informative to know the identity and functionality of all genes up/down regulated by such toxicants, this would appear a longer term goal, as the majority of human genes have not yet been sequenced, far less their functionality determined. However, the current use of gene profiling yields a *pattern* of gene changes for a xenobiotic of unknown toxicity which may be matched to that of well-characterized toxins, thus alerting the toxicologist to possible *in vivo* similarities between the unknown and the standard, thereby providing a platform for more extensive toxicological examination. Such approaches are beginning to gain momentum, in that several biotechnology companies are commercially producing 'gene chips' or 'gene arrays' that may be interrogated for toxicity assessment of xenobiotics. These chips consist of hundreds/thousands of genes, some of which are degenerate in the sense that not all of the genes are mechanistically-related to any one toxicological phenomenon. Whereas these chips are useful in broad-spectrum screening, they are maturing at a substantial rate, in that gene arrays are now becoming more specific, e.g. chips for the identification of changes in growth factor families that contribute to the aetiology and development of chemically-induced neoplasias.

Although documenting and explaining these genetic changes presents a formidable obstacle to understanding the different mechanisms of development and disease progression, the technology is now available to begin attempting this difficult challenge. Indeed, several 'differential expression analysis' methods have been developed which facilitate the identification of gene products that demonstrate

altered expression in cells of one population compared to another. These methods have been used to identify differential gene expression in many situations, including invading pathogenic microbes (Zhao *et al.* 1998), in cells responding to extracellular and intracellular microbial invasion (Duguid and Dinauer 1990, Ragno *et al.* 1997, Maldarelli *et al.* 1998), in chemically treated cells (Syed *et al.* 1997, Rockett *et al.* 1999), neoplastic cells (Liang *et al.* 1992, Chang and Terzaghi-Howe 1998), activated cells (Gurskaya *et al.* 1996, Wan *et al.* 1996), differentiated cells (Hara *et al.* 1991, Guimaraes *et al.* 1995a, b), and different cell types (Davis *et al.* 1984, Hedrick *et al.* 1984, Xhu *et al.* 1998). Although differential expression analysis technologies are applicable to a broad range of models, perhaps their most important advantage is that, in most cases, absolutely no prior knowledge of the specific genes which are up- or down-regulated is required.

The field of differential expression analysis is a large and complex one, with many techniques available to the potential user. These can be categorized into several methodological approaches, including:

- (1) Differential screening,
- (2) Subtractive hybridization (SH) (includes methods such as chemical cross-linking subtraction—CCLS, suppression-PCR subtractive hybridization—SSH, and representational difference analysis—RDA),
- (3) Differential display (DD),
- (4) Restriction endonuclease facilitated analysis (including serial analysis of gene expression—SAGE—and gene expression fingerprinting—GEF),
- (5) Gene expression arrays, and
- (6) Expressed sequence tag (EST) analysis.

The above approaches have been used successfully to isolate differentially expressed genes in different model systems. However, each method has its own subtle (and sometimes not so subtle) characteristics which incur various advantages and disadvantages. Accordingly, it is the purpose of this review to clarify the mechanistic principles underlying the main differential expression methods and to highlight some of the broader considerations and implications of this very powerful and increasingly popular technique. Specifically, we will concentrate on the so-called 'open' systems, namely those which do not require any knowledge of gene sequences and, therefore, are useful for isolating unknown genes. Two 'closed' systems (those utilising previously identified gene sequences), EST analysis and the use of DNA arrays, will also be considered briefly for completeness. Whilst emphasis will often be placed on suppression PCR subtractive hybridization (SSH, the approach employed in this laboratory), it is the aim of the authors to highlight, wherever possible, those areas of common interest to those who use, or intend to use, differential gene expression analysis.

Differential cDNA library screening (DS)

Despite the development of multiple technological advances which have recently brought the field of gene expression profiling to the forefront of molecular analysis, recognition of the importance of differential gene expression and characterization of differentially expressed genes has existed for many years. One of the original approaches used to identify such genes was described 20 years ago by St John and Davis (1979). These authors developed a method, termed 'differential plaque filter

hybridization', which was used to isolate galactose-inducible DNA sequences from yeast. The theory is simple: a genomic DNA library is prepared from normal, unstimulated cells of the test organism/tissue and multiple filter replicas are prepared. These replica blots are probed with radioactively (or otherwise) labelled complex cDNA probes prepared from the control and test cell mRNA populations. Those mRNAs which are differentially expressed in the treated cell population will show a positive signal only on the filter probed with cDNA from the treated cells. Furthermore, labelled cDNA from different test conditions can be used to probe multiple blots, thereby enabling the identification of mRNAs which are only up-regulated under certain conditions. For example, St John and Davis (1979) screened replica filters with acetate-, glucose- and galactose-derived probes in order to obtain genes induced specifically by galactose metabolism. Although groundbreaking in its time this method is now considered insensitive and time-consuming, as up to 2 months are required to complete the identification of genes which are differentially expressed in the test population. In addition, there is no convenient way to check that the procedure has worked until the whole process has been completed.

Subtractive Hybridization (SH)

The developing concept of differential gene expression and the success of early approaches such as that described by St John and Davis (1979) soon gave rise to a search for more convenient methods of analysis. One of the first to be developed was SH, numerous variations of which have since been reported (see below). In general, this approach involves hybridization of mRNA/cDNA from one population (tester) to excess mRNA/cDNA from another (driver), followed by separation of the unhybridized tester fraction (differentially expressed) from the hybridized common sequences. This step has been achieved physically, chemically and through the use of selective polymerase chain reaction (PCR) techniques.

Physical separation

Original subtractive hybridization technology involved the physical separation of hybridized common species from unique single stranded species. Several methods of achieving this have been described, including hydroxyapatite chromatography (Sargent and Dawid 1983), avidin-biotin technology (Duguid and Dinauer 1990) and oligodT-latex separation (Hara *et al.* 1991). In the first approach, common mRNA species are removed by cDNA (from test cells)-mRNA (from control cells) subtractive hybridization followed by hydroxyapatite chromatography, as hydroxyapatite specifically adsorbs the cDNA-mRNA hybrids. The unabsorbed cDNA is then used either for the construction of a cDNA library of differentially expressed genes (Sargent and Dawid 1983, Schneider *et al.* 1988) or directly as a probe to screen a preselected library (Zimmerman *et al.* 1980, Davis *et al.* 1984, Hedrick *et al.* 1984). A schematic diagram of the procedure is shown in figure 1.

Less rigorous physical separation procedures coupled with sensitivity enhancing PCR steps were later developed as a means to overcome some of the problems encountered with the hydroxyapatite procedure. For example, Duguid and Dinauer (1990) described a method of subtraction utilizing biotin-affinity systems as a means to remove hybridized common sequences. In this process, both the control and tester mRNA populations are first converted to cDNA and an adaptor ('oligovector',

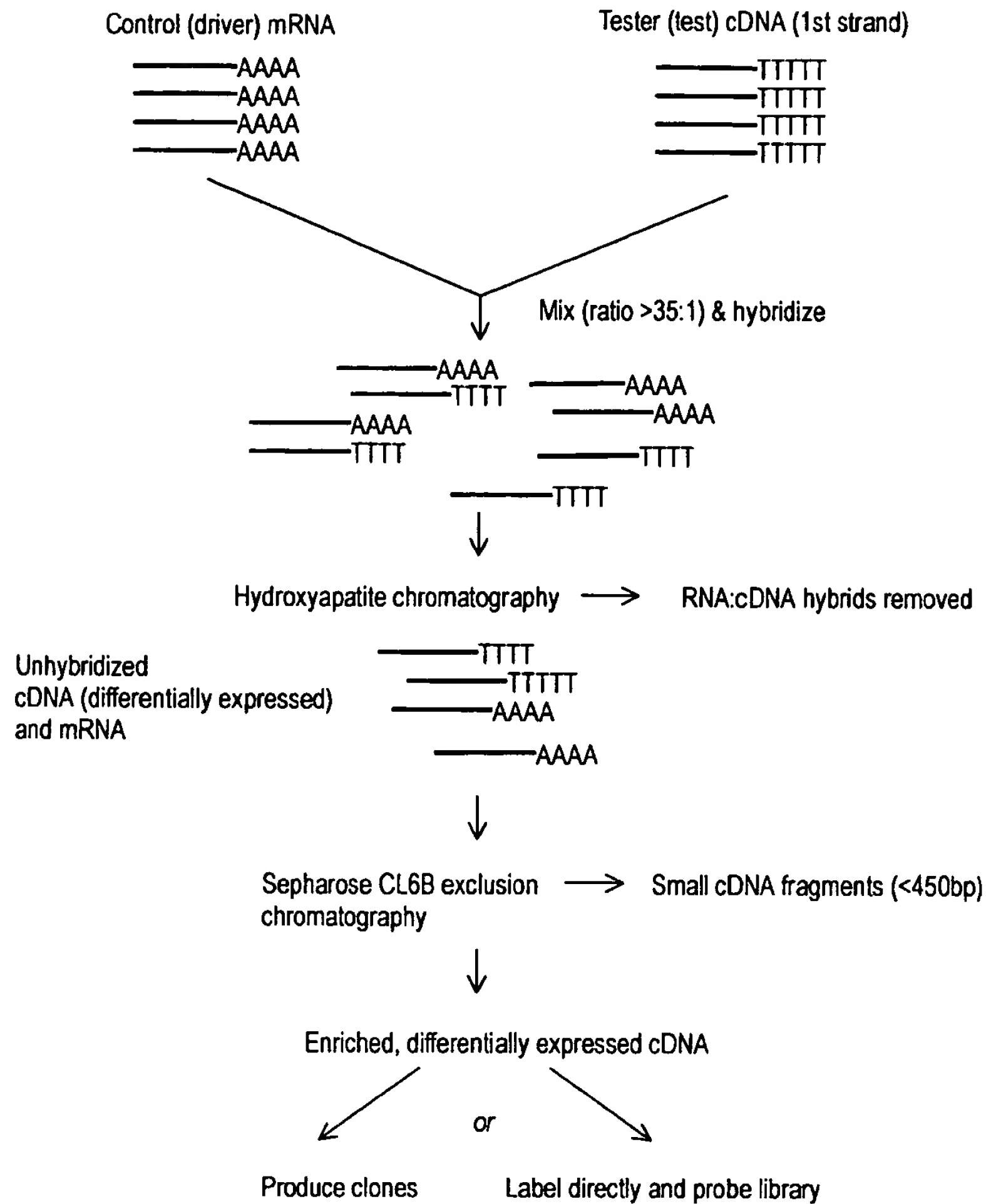


Figure 1. The hydroxyapatite method of subtractive hybridization. cDNA derived from the treated/alterd (tester) population is mixed with a large excess of mRNA from the control (driver) population. Following hybridization, mRNA-cDNA hybrids are removed by hydroxyapatite chromatography. The only cDNAs which remain are those which are differentially expressed in the treated/alterd population. In order to facilitate the recovery of full length clones, small cDNA fragments are removed by exclusion chromatography. The remaining cDNAs are then cloned into a vector for sequencing, or labelled and used directly to probe a library, as described by Sargent and Dawid (1983).

containing a restriction site) ligated to both sides. Both populations are then amplified by PCR, but the driver cDNA population is subsequently digested with the adaptor-containing restriction endonuclease. This serves to cleave the oligo-vector and reduce the amplification potential of the control population. The digested control population is then biotinylated and an excess mixed with tester cDNA. Following denaturation and hybridization, the mix is applied to a biocytin column (streptavidin may also be used) to remove the control population, including heteroduplexes formed by annealing of common sequences from the tester population. The procedure is repeated several times following the addition of fresh

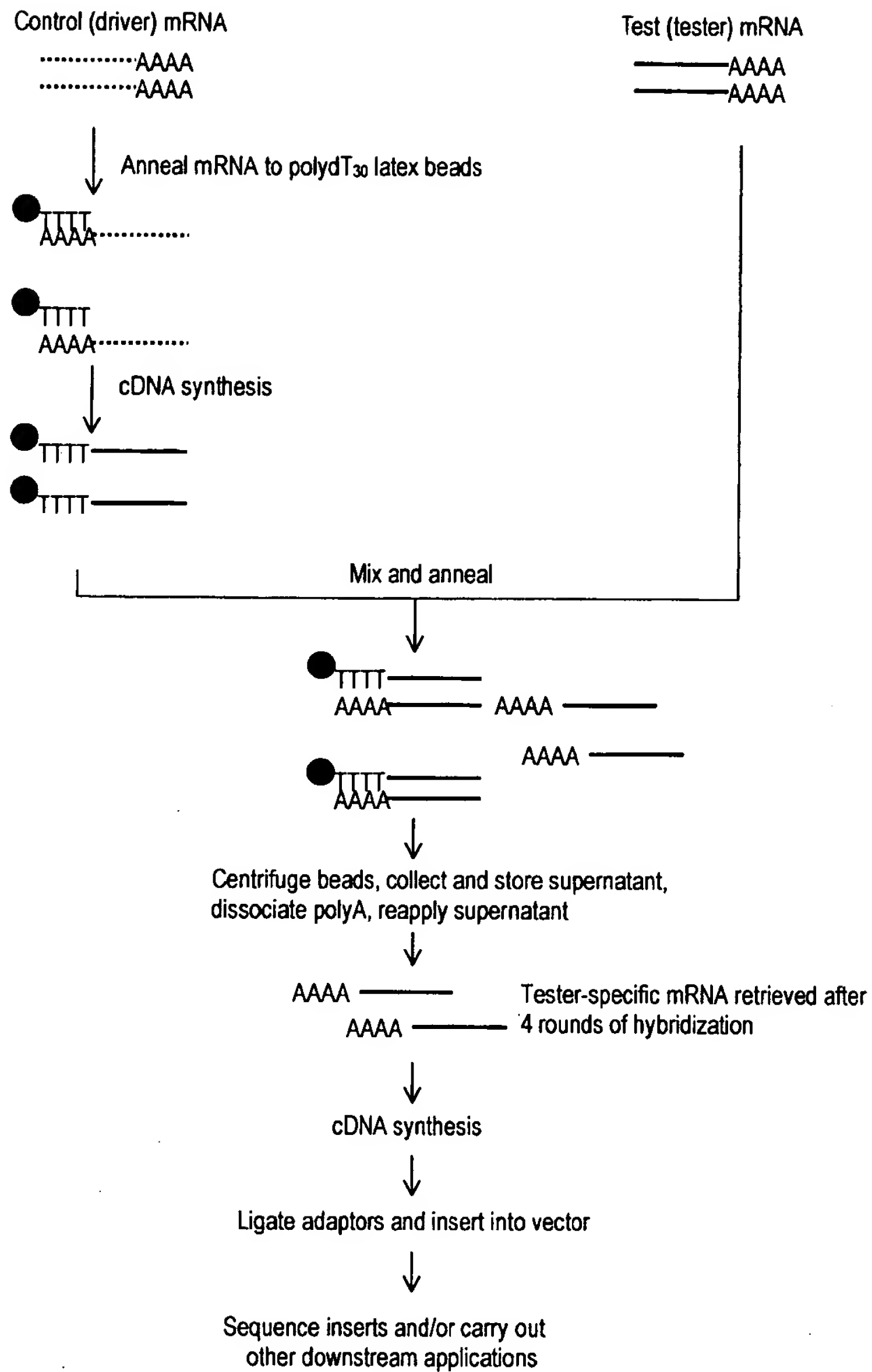


Figure 2. The use of oligodT₃₀ latex to perform subtractive hybridization. mRNA extracted from the control (driver) population is converted to anchored cDNA using polydT oligonucleotides attached to latex beads. mRNA from the treated/alterd (tester) population is repeatedly hybridized against an excess of the anchored driver cDNA. The final population of mRNA is tester specific and can be converted into cDNA for cloning and other downstream applications, as described by Hara *et al.* (1991).

control cDNA. In order to further enrich those species differentially expressed in the tester cDNA, the subtracted tester population is amplified by PCR following every second subtraction cycle. After six cycles of subtraction (three reamplification steps) the reaction mix is ligated into a vector for further analysis.

In a slightly different approach, Hara *et al.* (1991) utilized a method whereby oligo(dT₃₀) primers attached to a latex substrate are used to first capture mRNA extracted from the control population. Following 1st strand cDNA synthesis, the RNA strand of the heteroduplexes is removed by heat denaturation and centrifugation (the cDNA-oligotex-dT₃₀ forms a pellet and the supernatant is removed). A quantity of tester mRNA is then repeatedly hybridized to the immobilized control (driver) cDNA (which is present in 20-fold excess). After several rounds of hybridization the only mRNA molecules left in the tester mRNA population are those which are not found in the driver cDNA-oligotex-dT₃₀ population. These tester-specific mRNA species are then converted to cDNA and, following the addition of adaptor sequences, amplified by PCR. The PCR products are then ligated into a vector for further analysis using restriction sites incorporated into the PCR primers. A schematic illustration of this subtraction process is shown in figure 2.

However, all these methods utilising physical separation have been described as inefficient due to the requirement for large starting amounts of mRNA, significant loss of material during the separation process and a need for several rounds of hybridization. Hence, new methods of differential expression analysis have recently been designed to eliminate these problems.

Chemical Cross-Linking Subtraction (CCLS)

In this technique, originally described by Hampson *et al.* (1992), driver mRNA is mixed with tester cDNA (1st strand only) in a ratio of > 20:1. The common sequences form cDNA:mRNA hybrids, leaving the tester specific species as single stranded cDNA. Instead of physically separating these hybrids, they are inactivated chemically using 2,5 diaziridinyl-1,4-benzoquinone (DZQ). Labelled probes are then synthesized from the remaining single stranded cDNA species (unreacted mRNA species remaining from the driver are not converted into probe material due to specificity of Sequenase T7 DNA polymerase used to make the probe) and used to screen a cDNA library made from the tester cell population. A schematic diagram of the system is shown in figure 3.

It has been shown that the differentially expressed sequences can be enriched at least 300-fold with one round of subtraction (Hampson *et al.* 1992), and that the technique should allow isolation of cDNAs derived from transcripts that are present at less than 50 copies per cell. This equates to genes at the low end of intermediate abundance (see table 1). The main advantages of the CCLS approach are that it is rapid, technically simple and also produces fewer false positives than other differential expression analysis methods. However, like the physical separation protocols, a major drawback with CCLS is the large amount of starting material required (at least 10 µg RNA). Consequently, the technique has recently been refined so that a renewable source of RNA can be generated. The degenerate random oligonucleotide primed (DROP) adaptation (Hampson *et al.* 1996, Hampson and Hampson 1997) uses random hexanucleotide sequences to prime solid phase-synthesized cDNA. Since each primer includes a T7 polymerase promotor sequence

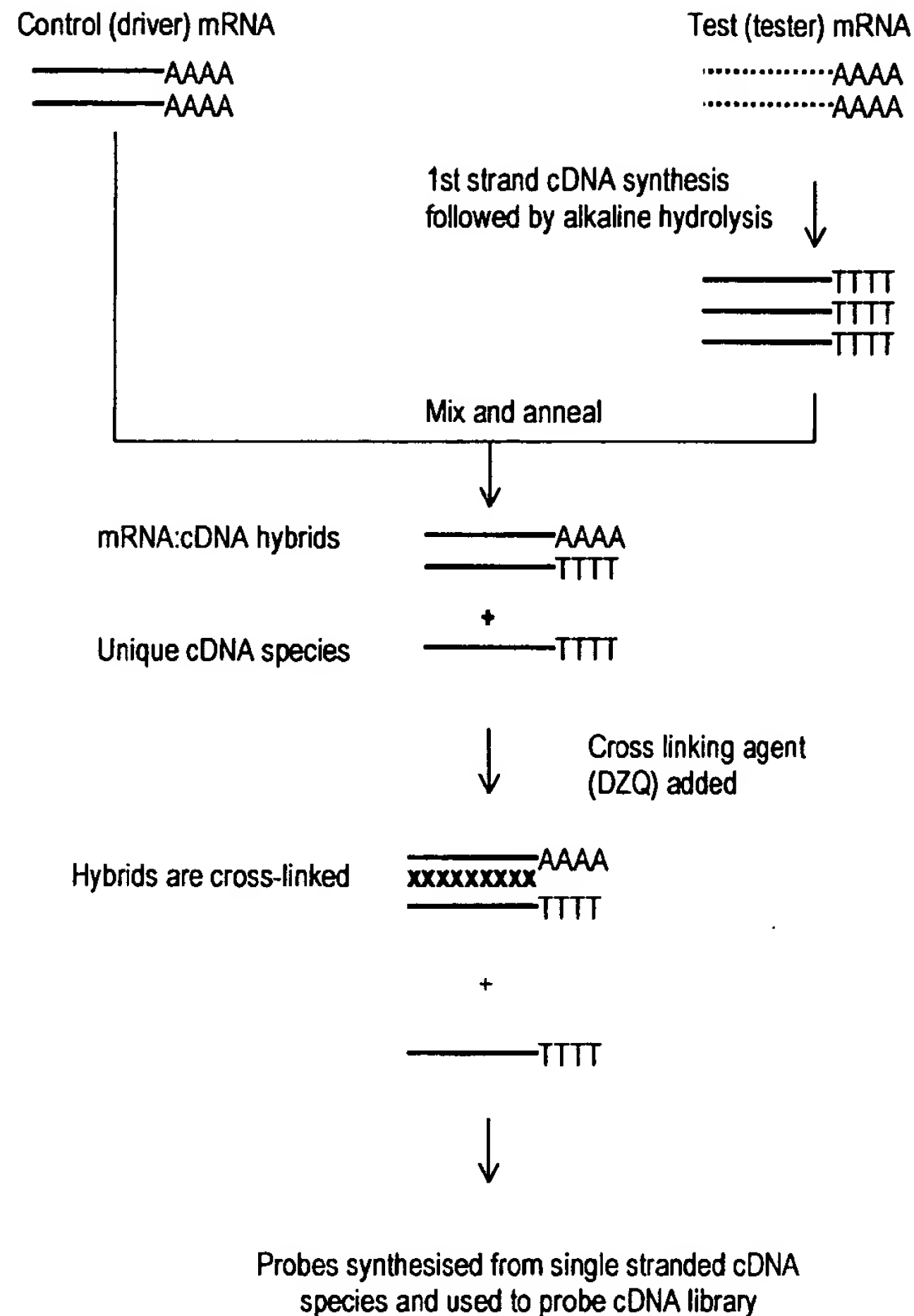


Figure 3. Chemical cross-linking subtraction. Excess driver mRNA is mixed with 1st strand tester cDNA. The common sequences form mRNA:cDNA hybrids which are cross linked with 2,5 diaziridinyl-1,4-benzoquinone (DZQ) and the remaining cDNA sequences are differentially expressed in the tester population. Probes are made from these sequences using Sequenase 2.0 DNA polymerase, which lacks reverse transcriptase activity and, therefore, does not react with the remaining mRNA molecules from the driver. The labelled probes are then used to screen a cDNA library for clones of differentially expressed sequences. Adapted from Walter *et al.* (1996), with permission.

Table 1. The abundance of mRNA species and classes in a typical mammalian cell.

| mRNA class | Copies of each species/cell | No. of mRNA species in class | Mean % of each species in class | Mean mass (ng) of each species/ μ g total RNA |
|--------------|-----------------------------|------------------------------|---------------------------------|---|
| Abundant | 12000 | 4 | 3.3 | 1.65 |
| Intermediate | 300 | 500 | 0.08 | 0.04 |
| Rare | 15 | 11000 | 0.004 | 0.002 |

Modified from Bertoli *et al.* (1995).

at the 5' end, the final pool of random cDNA fragments is a PCR-renewable cDNA population which is representative of the expressed gene pool and can be used to synthesize sense RNA for use as driver material. Furthermore, if the final pool of random cDNA fragments is reamplified using biotinylated T7 primer and random hexamer, the product can be captured with streptavidin beads and the antisense strand eluted for use as tester. Since both target and driver can be generated from the same DROP product, subtraction can be performed in both directions (i.e. for up- and down-regulated species) between two different DROP products.

Representational Difference Analysis (RDA)

RDA of cDNA (Hubank and Schatz 1994) is an extension of the technique originally applied to genomic DNA as a means of identifying differences between two complex genomes (Lisitsyn *et al.* 1993). It is a process of subtraction and amplification involving subtractive hybridization of the tester in the presence of excess driver. Sequences in the tester that have homologues in the driver are rendered unamplifiable, whereas those genes expressed only in the tester retain the ability to be amplified by PCR. The procedure is shown schematically in figure 4.

In essence, the driver and tester mRNA populations are first converted to cDNA and amplified by PCR following the ligation of an adaptor. The adaptors are then removed from both populations and a new (different) adaptor ligated to the amplified tester population only. Driver and tester populations are next melted and hybridized together in a ratio of 100:1. Following hybridization, only tester:tester homohybrids have 5' adaptors at each end of the DNA duplex and can, thus, be filled in at both 3' ends. Hence, only these molecules are amplified exponentially during the subsequent PCR step. Although tester:driver heterohybrids are present, they only amplify in a linear fashion, since the strand derived from the driver has no adaptor to which the primer can bind. Driver:driver heterohybrids have no adaptors and, therefore, are not amplified. Single stranded molecules are digested with mung bean nuclease before a further PCR-enrichment of the tester:tester homohybrids. The adaptors on the amplified tester population are then replaced and the whole process repeated a further two or three times using an increasing excess of driver (Hubank and Shatz used a tester:driver ratio of 1:400, 1:80000 and 1:800000 for the second, third and fourth hybridizations, respectively). Different adaptors are ligated to the tester between successive rounds of hybridization and amplification to prevent the accumulation of PCR products that might interfere with subsequent amplifications. The final display is a series of differentially expressed gene products easily observable on an ethidium bromide gel.

The main advantages of RDA are that it offers a reproducible and sensitive approach to the analysis of differentially expressed genes. Hubank and Schatz (1994) reported that they were able to isolate genes that were differentially expressed in substantially less than 1% of the cells from which the tester is derived. Perhaps the main drawback is that multiple rounds of ligation, hybridization, amplification and digestion are required. The procedure is, therefore, lengthier than many other differential display approaches and provides more opportunity for operator-induced error to occur. Although the generation of false positives has been noted, this has been solved to some degree by O'Neill and Sinclair (1997) through the use of HPLC-purified adaptors. These are free of the truncated adaptors which appear to be a major source of the false positive bands. A very similar technique to RDA, termed linker capture subtraction (LCS) was described by Yang and Sytowski (1996).

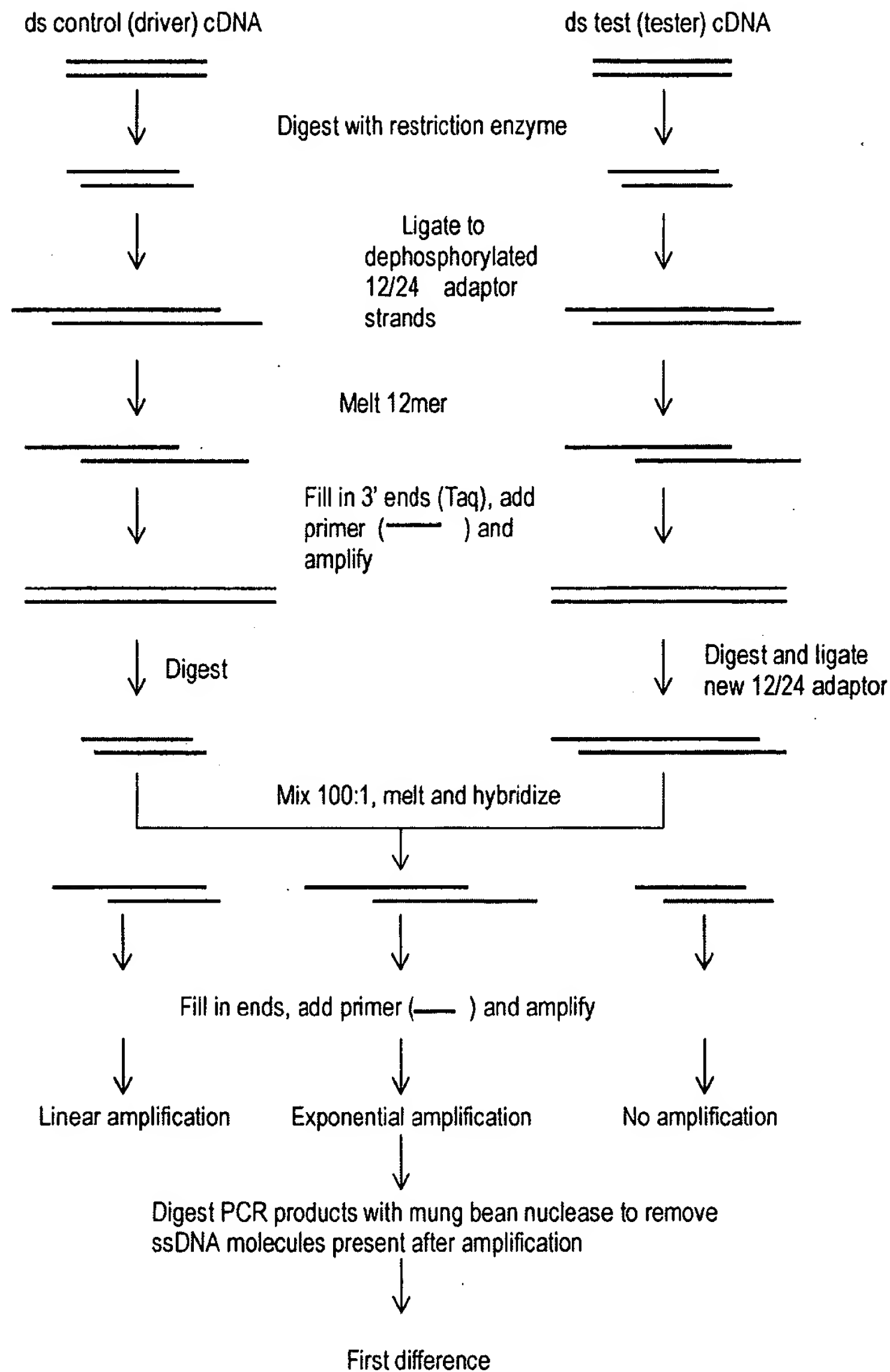


Figure 4. The representational difference analysis (RDA) technique. Driver and tester cDNA are digested with a 4-cutter restriction enzyme such as *DpnII*. The 1st set of 12/24 adaptor strands (oligonucleotides) are ligated to each other and the digested cDNA products. The 12mer is subsequently melted away and the 3' ends filled in using Taq DNA polymerase. Each cDNA population is then amplified using PCR, following which the 1st set of adaptors is removed with *DpnII*. A second set of 12/24 adaptor strands is then added to the amplified tester cDNA population, after which the tester is hybridized against a large excess of driver. The 12mer adaptors are melted and the 3' ends filled in as before. PCR is carried out with primers identical to the new 24mer adaptor. Thus, the only hybridization products which are exponentially amplified are those which are tester:tester combinations. Following PCR, ssDNA products are removed with mung bean nuclease, leaving the 'first difference product'. This is digested and a third set of 12/24 adaptors added before repeating the subtraction process from the hybridization stage. The process is repeated to the 3rd or 4th difference product, as described by Lisitsyn *et al.* (1993) and Hubank and Schatz (1994).

Suppression PCR Subtractive Hybridization (SSH)

The most recent adaptation of the SH approach to differential expression analysis was first described by Diatchenko *et al.* (1996) and Gurskaya *et al.* (1996). They reported that a 1000–5000 fold enrichment of rare cDNAs (equivalent to isolating mRNAs present at only a few copies per cell) can be obtained without the need for multiple hybridizations/subtractions. Instead of physical or chemical removal of the common sequences, a PCR-based suppression system is used (see figure 5).

In SSH, excess driver cDNA is added to two portions of the tester cDNA which have been ligated with different adaptors. A first round of hybridization serves to enrich differentially expressed genes and equalize rare and abundant messages. Equalization occurs since reannealing is more rapid for abundant molecules than for rarer molecules due to the second order kinetics of hybridization (James and Higgins 1985). The two primary hybridization mixes are then mixed together in the presence of excess driver and allowed to hybridize further. This step permits the annealing of single stranded complementary sequences which did not hybridize in the primary hybridization, and in doing so generates templates for PCR amplification. Although there are several possible combinations of the single stranded molecules present in the secondary hybridization mix, only one particular combination (differentially expressed in the tester cDNA composed of complementary strands having different adaptors) can amplify exponentially.

Having obtained the final differential display, two options are available if cloning of cDNAs is desired. One is to transform the whole of the final PCR reaction into competent cells. Transformed colonies can then be isolated and their inserts characterized by sequencing, restriction analysis or PCR. Alternatively, the final PCR products can be resolved on a gel and the individual bands excised, reamplified and cloned. The first approach is technically simpler and less time consuming. However, ligation/transformation reactions are known to be biased towards the cloning of smaller molecules, and so the final population of clones will probably not contain a representative selection of the larger products. In addition, although equalization theoretically occurs, observations in this laboratory suggest that this is by no means perfectly accomplished. Consequently, some gene species are present in a higher number than others and this will be represented in the final population of clones. Thus, in order to obtain a substantial proportion of those gene species that actually demonstrate differential expression in the tester population, the number of clones that will have to be screened after this step may be substantial. The second approach is initially more time consuming and technically demanding. However, it would appear to offer better prospects for cloning larger and low abundance gel products. In addition, one can incorporate a screening step that differentiates different products of different sequences but of the same size (HA-staining, see later). In this way, a good idea of the final number of clones to be isolated and identified can be achieved.

An alternative (or even complementary) approach is to use the final differential display reaction to screen a cDNA library to isolate full length clones for further characterization, or a DNA array (see later) to quickly identify known genes. SSH has been used in this laboratory to begin characterization of the short-term gene expression profiles of enzyme-inducers such as phenobarbital (Rockett *et al.* 1997) and Wy-14,643 (Rockett *et al.* unpublished observations). The isolation of differentially expressed genes in this manner enables the construction of a fingerprint

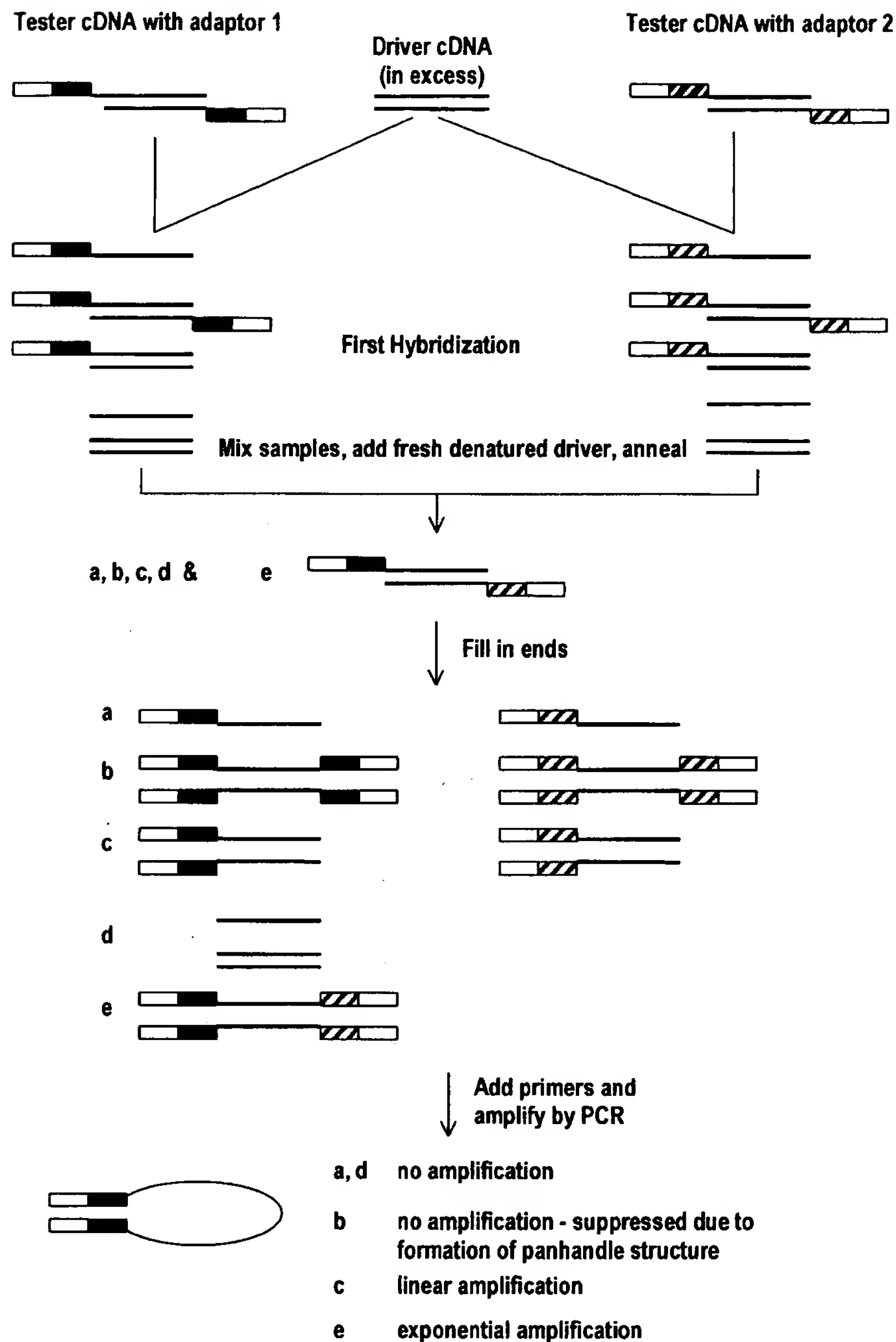


Figure 5. PCR-select cDNA subtraction. In the primary hybridization, an excess of driver cDNA is added to each tester cDNA population. The samples are heat denatured and allowed to hybridize for between 3 and 8 h. This serves two purposes: (1) to equalize rare and abundant molecules; and (2) to enrich for differentially expressed sequences—cDNAs that are not differentially expressed form type c molecules with the driver. In the secondary hybridization, the two primary hybridizations are mixed together without denaturing. Fresh denatured driver can also be added at this point to allow further enrichment of differentially expressed sequences. Type e molecules are formed in this secondary hybridization which are subsequently amplified using two rounds of PCR. The final products can be visualized on an agarose gel, labelled directly or cloned into a vector for downstream manipulation. As described by Diatchenko *et al.* (1996) and Gurskaya *et al.* (1996), with permission.

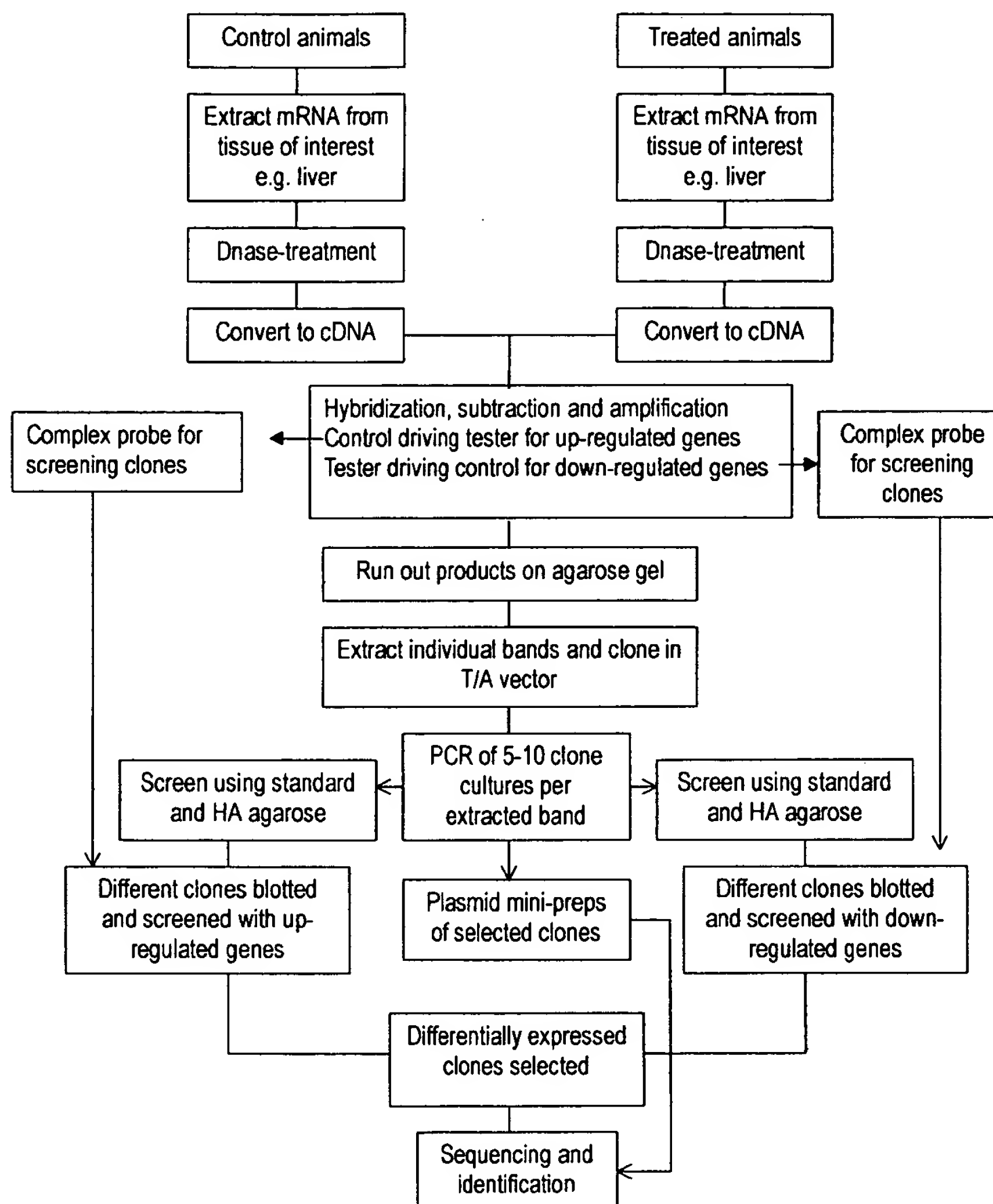


Figure 6. Flow diagram showing method used in this laboratory to isolate and identify clones of genes which are differentially expressed in rat liver following short term exposure to the enzyme inducers, phenobarbital and Wy-14,643.

of expressed genes which are unique to each compound and time/dose point. Such information could be useful in short-term characterization of the toxic potential of new compounds by comparing the gene-expression profiles they elicit with those produced by known inducers. Figure 6 shows a flow diagram of the method used to isolate, verify and clone differentially expressed genes, and figure 7 shows expression profiles obtained from a typical SSH experiment. Subsequent sub-cloning of the individual bands, sequencing and gene data base interrogation reveals many genes which are either up- or down-regulated by phenobarbital in the rat (tables 2 and 3).

One of the advantages in using the SSH approach is that no prior knowledge is required of which specific genes are up/down-regulated subsequent to xenobiotic

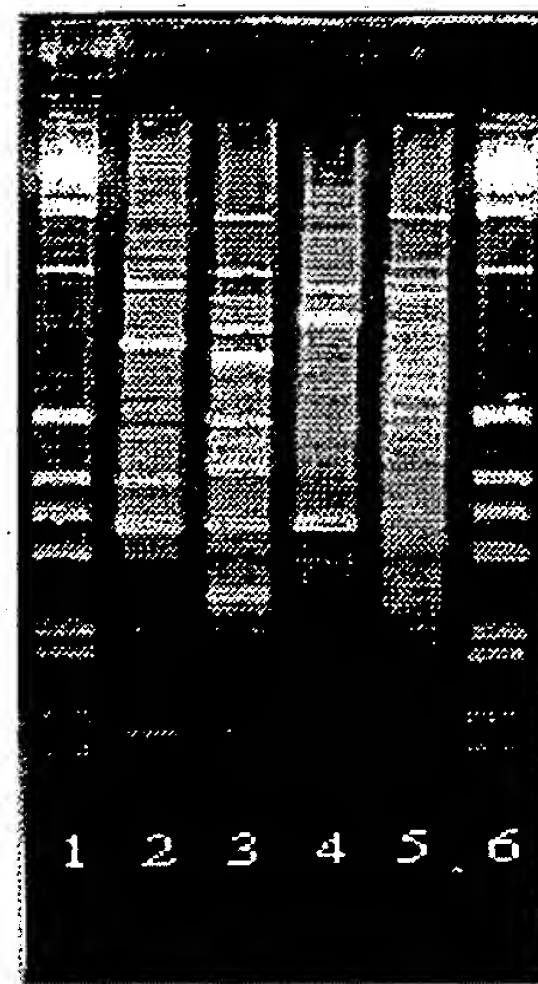


Figure 7. SSH display patterns obtained from rat liver following 3-day treatment with WY-14,643 or phenobarbital. mRNA extracted from control and treated livers was used to generate the differential displays using the PCR-Select cDNA subtraction kit (Clontech). Lane: 1—1kb ladder; 2—genes upregulated following Wy,14-643 treatment; 3—genes downregulated following Wy,14-643 treatment; 4—genes upregulated following phenobarbital treatment; 5—genes downregulated following phenobarbital treatment; 6—1kb ladder. Reproduced from Rockett *et al.* (1997), with permission.

exposure, and an almost complete complement of genes are obtained. For example, the peroxisome proliferator and non-genotoxic hepatocarcinogen Wy,14,643, up-regulates at least 28 genes and down-regulates at least 15 in the rat (a sensitive species) and produces 48 up- and 37 down-regulated genes in the guinea pig, a resistant species (Rockett, Swales, Esda and Gibson, unpublished observations). One of these genes, CD81, was up-regulated in the rat and down-regulated in the guinea pig following Wy-14,643 treatment. CD81 (alternatively named TAPA-1) is a widely expressed cell surface protein which is involved in a large number of cellular processes including adhesion, activation, proliferation and differentiation (Levy *et al.* 1998). Since all of these functions are altered to some extent in the phenomena of hepatomegaly and non-genotoxic hepatocarcinogenesis, it is intriguing, and probably mechanistically-relevant, that CD81 expression is differentially regulated in a resistant and susceptible species. However, the down-side of this approach is that the majority of genes can be sequenced and matched to database sequences, but the latter are predominantly expressed sequence tags or genes of completely unknown function, thus partially obscuring a realistic overall assessment of the critical genes of genuine biological interest. Notwithstanding the lack of complete functional identification of altered gene expression, such gene profiling studies essentially provides a 'molecular fingerprint' in response to xenobiotic challenge, thereby serving as a mechanistically-relevant platform for further detailed investigations.

Differential Display (DD)

Originally described as 'RNA fingerprinting by arbitrarily primed PCR' (Liang and Pardee 1992) this method is now more commonly referred to as 'differential

Table 2. Genes up-regulated in rat liver following 3-day exposure to phenobarbital.

| Band number (approximate size in bp) | Highest sequence similarity | FASTA-EMBL gene identification |
|--|--------------------------------|--------------------------------------|
| 5 (1300) | 93.5% | CYP2B1 |
| 7 (1000) | 95.1% | Preproalbumin Serum albumin mRNA |
| 8 (950) | 98.3% | NCI-CGAP-Pr1 <i>H. sapiens</i> (EST) |
| 10 (850) | 95.7% | CYP2B1 |
| 11 (800) | Clone 1 94.9% | CYP2B1 |
| | Clone 2 75.3% | CYP2B2 |
| 12 (750) | 93.8% | TRPM-2 mRNA Sulfated glycoprotein |
| 15 (600) | 92.9% | Preproalbumin Serum albumin mRNA |
| 16 (55) | Clone 1 95.2% | CYP2B1 |
| | Clone 2 93.6% | Haptoglobulin mRNA partial alpha |
| 21 (350) | 99.3% | 18S, 5.8S & 28S rRNA |

Bands 1–4, 6, 9, 13, 14, and 17–20 are shown to be false positives by dot blot analysis and, therefore, are not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are up-regulated in rat liver by phenobarbital, but simply represents the genes sequenced and identified to date.

Table 3. Genes down-regulated in rat liver following 3-day exposure to phenobarbital.

| Band number (approximate size in bp) | Highest sequence similarity | FASTA-EMBL gene identification |
|--|--------------------------------|---|
| 1 (1500) | 95.3% | 3-oxoacyl-CoA thiolase |
| 2 (1200) | 92.3% | Hemopoxin mRNA |
| 3 (1000) | 91.7% | Alpha-2u-globulin mRNA |
| 7 (700) | Clone 1 77.2% | <i>M. musculus</i> C1 inhibitor |
| | Clone 2 94.5% | Electron transfer flavoprotein |
| | Clone 3 91.0% | <i>M. musculus</i> Topoisomerase 1 (Topo 1) |
| 8 (650) | Clone 1 86.9% | Soares 2NbMT <i>M. musculus</i> (EST) |
| | Clone 2 96.2% | Alpha-2u-globulin (s-type) mRNA |
| 9 (600) | Clone 1 86.9% | Soares mouse NML <i>M. musculus</i> (EST) |
| | Clone 2 82.0% | Soares p3NMF 19.5 <i>M. musculus</i> (EST) |
| 10 (550) | 73.8% | Soares mouse NML <i>M. musculus</i> (EST) |
| 11 (525) | 95.7% | NCI-CGAP-Pr1 <i>H. sapiens</i> (EST) |
| 12 (375) | 100.0% | Ribosomal protein |
| 13 (23) | Clone 1 97.2% | Soares mouse embryo NbME135 (EST) |
| | Clone 2 100.0% | Fibrinogen B-beta-chain |
| | Clone 3 100.0% | Apolipoprotein E gene |
| 14 (170) | 96.0% | Soares p3NMF19.5 <i>M. musculus</i> (EST) |
| 15 (140) | 97.3% | Stratagene mouse testis (EST) |
| Others: (300) | 96.7% | <i>R. norvegicus</i> RASP 1 mRNA |
| (275) | 93.1% | Soares mouse mammary gland (EST) |

EST = Expressed sequence tag. Bands 4–6 were shown to be false positives by dot blot analysis and, therefore, were not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are down-regulated in rat liver by phenobarbital, but simply represents the genes sequenced and identified to date.

display' (DD). In this method, all the mRNA species in the control and treated cell populations are amplified in separate reactions using reverse transcriptase-PCR (RT-PCR). The products are then run side-by-side on sequencing gels. Those bands which are present in one display only, or which are much more intense in one

display compared to the other, are differentially expressed and may be recovered for further characterization. One advantage of this system is the speed with which it can be carried out—2 days to obtain a display and as little as a week to make and identify clones.

Two commonly used variations are based on different methods of priming the reverse transcription step (figure 8). One is to use an oligo dT with a 2-base 'anchor' at the 3'-end, e.g. 5' (dT₁₁)CA 3' (Liang and Pardee 1992). Alternatively, an arbitrary primer may be used for 1st strand cDNA synthesis (Welsh *et al.* 1992). This variant of RNA fingerprinting has also been called 'RAP' (RNA Arbitrarily Primed)-PCR. One advantage of this second approach is that PCR products may be derived from anywhere in the RNA, including open reading frames. In addition, it can be used for mRNAs that are not polyadenylated, such as many bacterial mRNAs (Wong and McClelland 1994). In both cases, following reverse transcription and denaturation, second strand cDNA synthesis is carried out with an arbitrary primer (*arbitrary* primers have a single base at each position, as compared to *random* primers, which contain a mixture of all four bases at each position). The resulting PCR, thus, produces a series of products which, depending on the system (primer length and composition, polymerase and gel system), usually includes 50–100 products per primer set (Band and Sager 1989). When a combination of different dT-anchors and arbitrary primers are used, almost all mRNA species from a cell can be amplified. When the cDNA products from two different populations are analysed side by side on a polyacrylamide gel, differences in expression can be identified and the appropriate bands recovered for cloning and further analysis.

Although DD is perhaps the most popular approach used today for identifying differentially expressed genes, it does suffer from several perceived disadvantages:

- (1) It may have a strong bias towards high copy number mRNAs (Bertioli *et al.* 1995), although this has been disputed (Wan *et al.* 1996) and the isolation of very low abundance genes may be achieved in certain circumstances (Guimeraes *et al.* 1995a).
- (2) The cDNAs obtained often only represent the extreme 3' end of the mRNA (often the 3'-untranslated region), although this may not always be the case (Guimeraes *et al.* 1995a). Since the 3' end is often not included in Genbank and shows variation between organisms, cDNAs identified by DD cannot always be matched with their genes, even if they have been identified.
- (3) The pattern of differential expression seen on the display often cannot be reproduced on Northern blots, with false positives arising in up to 70% of cases (Sun *et al.* 1994). Some adaptations have been shown to reduce false positives, including the use of two reverse transcriptases (Sung and Denman 1997), comparison of uninduced and induced cells over a time course (Burn *et al.* 1994) and comparison of DDPCR-products from two uninduced and two induced lines (Sompayrac *et al.* 1995). The latter authors also reported that the use of cytoplasmic RNA rather than total RNA reduces false positives arising from nuclear RNA that is not transported to the cytoplasm.

Further details of the background, strengths and weaknesses of the DD technique can be obtained from a review by McClelland *et al.* (1996) and from articles by Liang *et al.* (1995) and Wan *et al.* (1996).

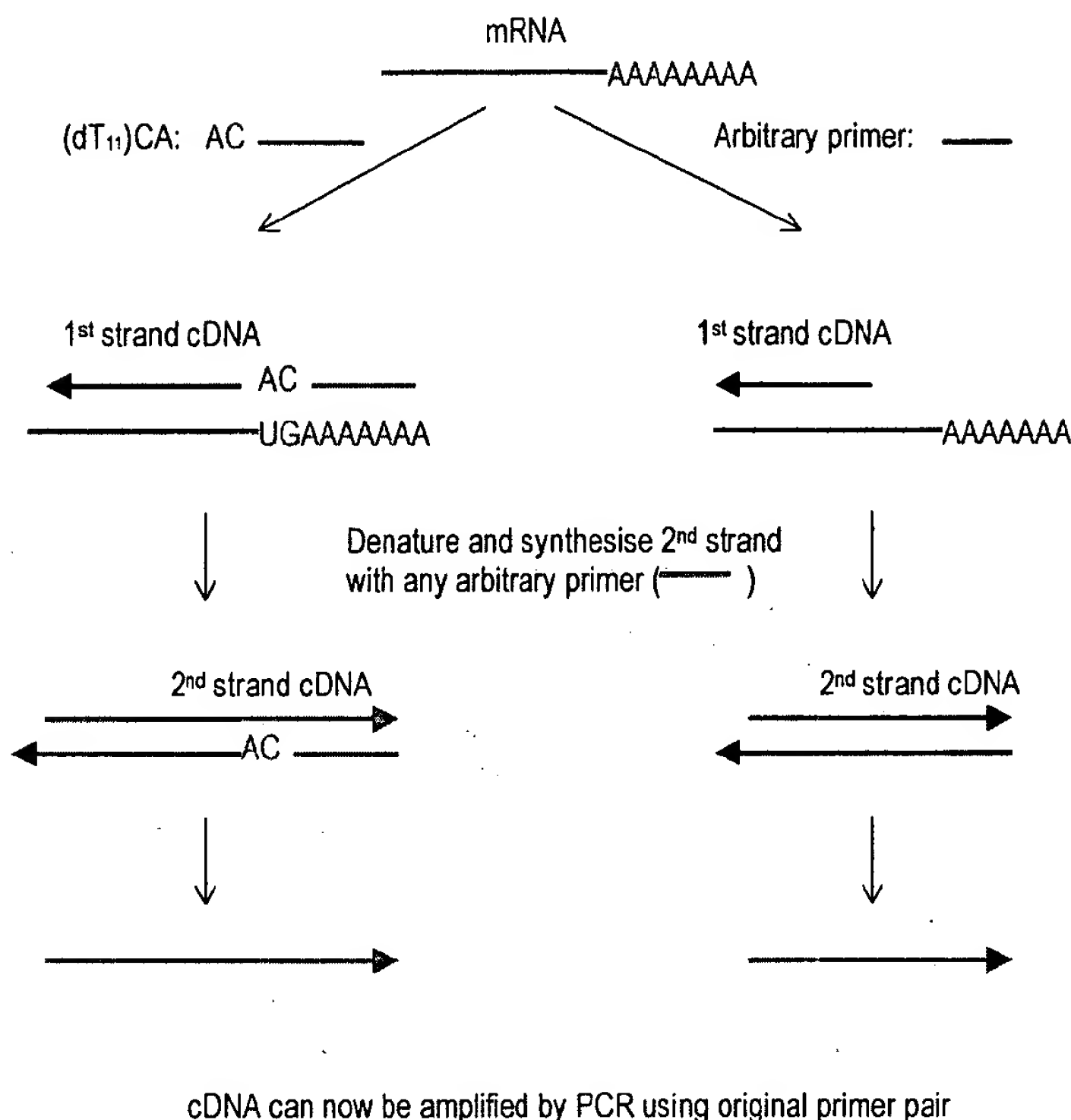


Figure 8. Two approaches to differential display (DD) analysis. 1st strand synthesis can be carried out either with a polyd(T)₁₁NN primer (where N = G, C or A) or with an arbitrary primer. The use of different combinations of G, C and A to anchor the first strand polyd(T) primer enables the priming of the majority of polyadenylated mRNAs. Arbitrary primers may hybridize at none, one or more places along the length of the mRNA, allowing 1st strand cDNA synthesis to occur at none, one or more points in the same gene. In both cases, 2nd strand synthesis is carried out with an arbitrary primer. Since these arbitrary primers for the 2nd strand may also hybridize to the 1st strand cDNA in a number of different places, several different 2nd strand products may be obtained from one binding point of the 1st strand primer. Following 2nd strand synthesis, the original set of primers is used to amplify the second strand products, with the result that numerous gene sequences are amplified.

Restriction endonuclease-facilitated analysis of gene expression

Serial Analysis of Gene Expression (SAGE)

A more recent development in the field of differential display is SAGE analysis (Velculescu *et al.* 1995). This method uses a different approach to those discussed so far and is based on two principles. Firstly, in more than 95% of cases, short nucleotide sequences ('tags') of only nine or 10 base pairs provide sufficient information to identify their gene of origin. Secondly, concatenation (linking together in a series) of these tags allows sequencing of multiple cDNAs within a single clone. Figure 9 shows a schematic representation of the SAGE process. In this procedure, double stranded cDNA from the test cells is synthesized with a biotinylated polyd(T) primer. Following digestion with a commonly cutting (4bp recognition sequence) restriction enzyme ('anchoring enzyme'), the 3' ends of the cDNA population are captured with streptavidin beads. The captured population is

split into two and different adaptors ligated to the 5' ends of each group. Incorporated into the adaptors is a recognition sequence for a type IIS restriction enzyme—one which cuts DNA at a defined distance (< 20 bp) from its recognition sequence. Hence, following digestion of each captured cDNA population with the IIS enzyme, the adaptors plus a short piece of the captured cDNA are released. The two populations are then ligated and the products amplified. The amplified products are cleaved with the original anchoring enzyme, religated (concatomers are formed in the process) and cloned. The advantage of this system is that hundreds of gene tags can be identified by sequencing only a few clones. Furthermore, the number of times a given transcript is identified is a quantitative measurement of that gene's abundance in the original population, a feature which facilitates identification of differentially expressed genes in different cell populations.

Some disadvantages of SAGE analysis include the technical difficulty of the method, a large amount of accurate sequencing is required, biased towards abundant mRNAs, has not been validated in the pharmaco/toxicogenomic setting and has only been used to examine well known tissue differences to date.

Gene Expression Fingerprinting (GEF)

A different capture/restriction digest approach for isolating differentially expressed genes has been described by Ivanova and Belyavsky (1995). In this method, RNA is converted to cDNA using biotinylated oligo(dT) primers. The cDNA population is then digested with a specific endonuclease and captured with magnetic streptavidin microbeads to facilitate removal of the unwanted 5' digestion products. The use of restricted 3'-ends alone serves to reduce the complexity of the cDNA fragment pool and helps to ensure that each RNA species is represented by not more than one restriction product. An adaptor is ligated to facilitate subsequent amplification of the captured population. PCR is carried out with one adaptor-specific and one biotinylated polydT primer. The reamplified population is recaptured and the non-biotinylated strands removed by alkaline dissociation. The non-biotinylated strand is then resynthesized using a different adaptor-specific primer in the presence of a radiolabelled dNTP. The labelled immobilized 3' cDNA ends are next sequentially treated with a series of different restriction endonucleases and the products from each digestion analysed by PAGE. The result is a fingerprint composed of a number of ladders (equal to the number of sequential digests used). By comparing test versus control fingerprints, it is possible to identify differentially expressed products which can then be isolated from the gel and cloned. The advantages of this procedure are that it is very robust and reproducible, and the authors estimate that 80–93% of cDNA molecules are involved in the final fingerprint. The disadvantage is that polyacrylamide gels can rarely resolve more than 300–400 bands, which compares poorly to the 1000 or more which are estimated to be produced in an average experiment. The use of 2-D gels such as those described by Uitterlinden *et al.* (1989) and Hatada *et al.* (1991) may help to overcome this problem.

A similar method for displaying restriction endonuclease fragments was later described by Prashar and Weissman (1996). However, instead of sequential digestion of the immobilized 3'-terminal cDNA fragments, these authors simply compared the profiles of the control and treated populations without further manipulation.

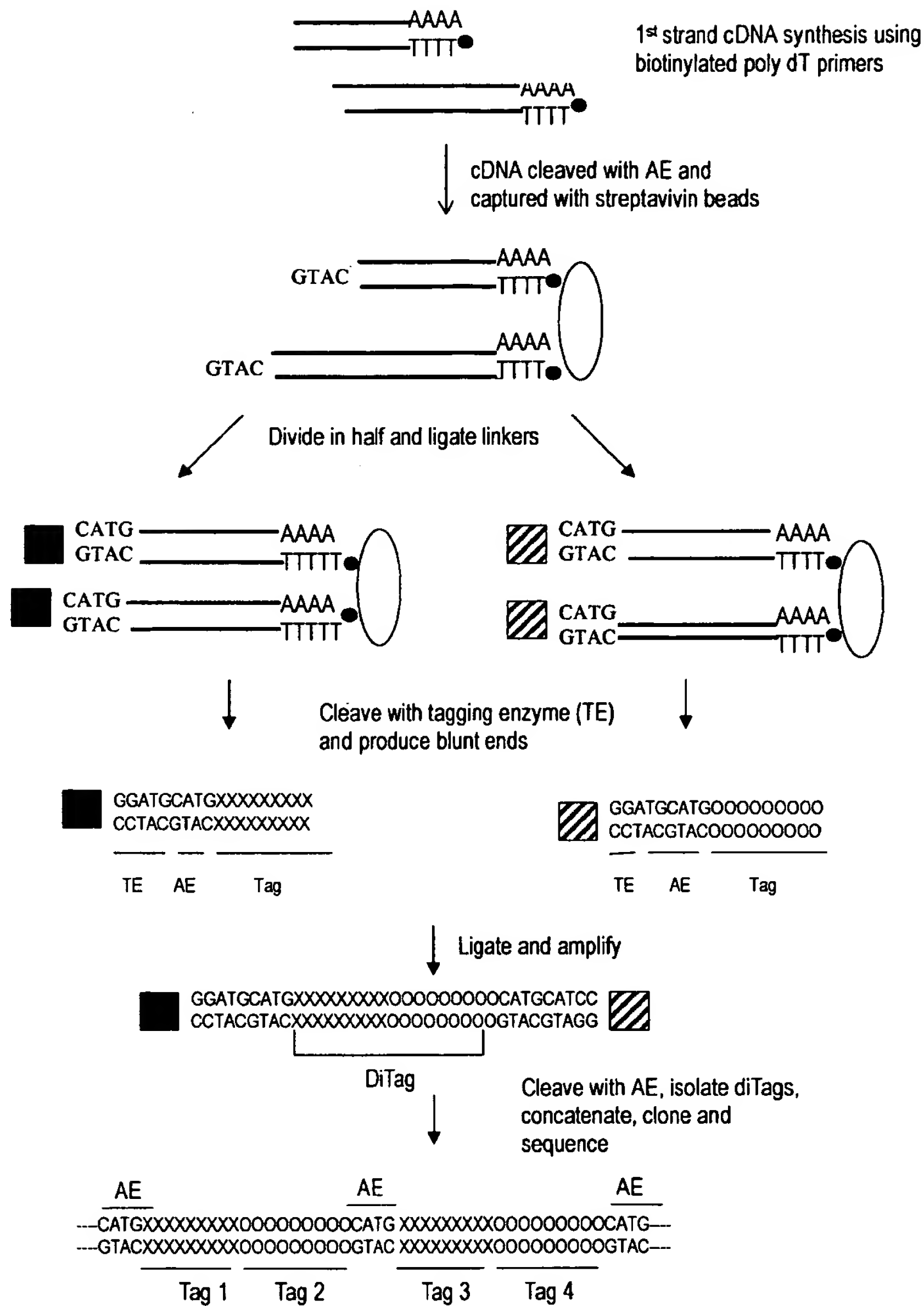


Figure 9. Serial analysis of gene expression (SAGE) analysis. cDNA is cleaved with an anchoring enzyme (AE) and the 3' ends captured using streptavidin beads. The cDNA pool is divided in half and each portion ligated to a different linker, each containing a type IIS restriction site (tagging enzyme, TE). Restriction with the type IIS enzyme releases the linker plus a short length of cDNA (XXXXXX and OOOOO indicate nucleotides of different tags). The two pools of tags are then ligated and amplified using linker-specific primers. Following PCR, the products are cleaved with the AE and the diTags isolated from the linkers using PAGE. The diTags are then ligated (during which process, concatenation occurs) and cloned into a vector of choice for sequencing. After Velculescu *et al.* (1995), with permission.

DNA arrays

'Open' differential display systems are cumbersome in that it takes a great deal of time to extract and identify candidate genes and then confirm that they are indeed up- or down-regulated in the treated compared to the control tissue. Normally, the latter process is carried out using Northern blotting or RT-PCR. Even so, each of the aforementioned steps produce a bottleneck to the ultimate goal of rapid analysis of gene expression. These problems will likely be addressed by the development of so-called DNA arrays (e.g. Gress *et al.* 1992, Zhao *et al.* 1995, Schena *et al.* 1996), the introduction of which has signalled the next era in differential gene expression analysis. DNA arrays consist of a gridded membrane or glass 'chips' containing hundreds or thousands of DNA spots, each consisting of multiple copies of part of a known gene. The genes are often selected based on previously proven involvement in oncogenesis, cell cycling, DNA repair, development and other cellular processes. They are usually chosen to be as specific as possible for each gene and animal species. Human and mouse arrays are already commercially available and a few companies will construct a personalized array to order, for example Clontech Laboratories and Research Genetics Inc. The technique is rapid in that hundreds or even thousands of genes can be spotted on a single array, and that mRNA/cDNA from the test populations can be labelled and used directly as probe. When analysed with appropriate hardware and software, arrays offer a rapid and quantitative means to assess differences in gene expression between two cell populations. Of course, there can only be identification and quantitation of those genes which are in the array (hence the term 'closed' system). Therefore, one approach to elucidating the molecular mechanisms involved in a particular disease/development system may be to combine an open and closed system—a DNA array to directly identify and quantitate the expression of known genes in mRNA populations, and an open system such as SSH to isolate unknown genes which are differentially expressed.

One of the main advantages of DNA arrays is the huge number of gene fragments which can be put on a membrane—some companies have reported gridding up to 60000 spots on a single glass 'chip' (microscope slide). These high density chip-based micro-arrays will probably become available as mass-produced off-the-shelf items in the near future. This should facilitate the more rapid determination of differential expression in time and dose-response experiments. Aside from their high cost and the technical complexities involved in producing and probing DNA arrays, the main problem which remains, especially with the newer micro-array (gene-chip) technologies, is that results are often not wholly reproducible between arrays. However, this problem is being addressed and should be resolved within the next few years.

EST databases as a means to identify differentially expressed genes

Expressed sequence tags (ESTs) are partial sequences of clones obtained from cDNA libraries. Even though most ESTs have no formal identity (putative identification is the best to be hoped for), they have proven to be a rapid and efficient means of discovering new genes and can be used to generate profiles of gene-expression in specific cells. Since they were first described by Adams *et al.* (1991), there has been a huge explosion in EST production and it is estimated that there are now well over a million such sequences in the public domain, representing over half

of all human genes (Hillier *et al.* 1996). This large number of freely available sequences (both sequence information and clones are normally available royalty-free from the originators) has enabled the development of a new approach towards differential gene expression analysis as described by Vasmatazis *et al.* (1998). The approach is simple in theory: EST databases are first searched for genes that have a number of related EST sequences from the target tissue of choice, but none or few from non-target tissue libraries. Programmes to assist in the assembly of such sets of overlapping data may be developed in-house or obtained privately or from the internet. For example, the Institute for Genomic Research (TIGR, found at <http://www.tigr.org>) provides many software tools free of charge to the scientific community. Included amongst these is the TIGR assembler (Sutton *et al.* 1995), a tool for the assembly of large sets of overlapping data such as ESTs, bacterial artificial chromosomes (BAC)s, or small genomes. Candidate EST clones representing different genes are then analysed using RNA blot methods for size and tissue specificity and, if required, used as probes to isolate and identify the full length cDNA clone for further characterization. In practice however, the method is rather more involved, requiring bioinformatic and computer analysis coupled with confirmatory molecular studies. Vasmatazis *et al.* (1998) have described several problems in this fledgling approach, such as separating highly homologous sequences derived from different genes and an overemphasis of specificity for some EST sequences. However, since these problems will largely be addressed by the development of more suitable computer algorithms and an increased completeness of the EST database, it is likely that this approach to identifying differentially expressed genes may enjoy more patronage in the future.

Problems and potential of differential expression techniques

The holistic or single cell approach?

When working with *in vivo* models of differential expression, one of the first issues to consider must be the presence of multiple cell types in any given specimen. For example, a liver sample is likely to contain not only hepatocytes, but also (potentially) Ito cells, bile ductule cells, endothelial cells, various immune cells (e.g. lymphocytes, macrophages and Kupffer cells) and fibroblasts. Other tissues will each have their own distinctive cell populations. Also, in the case of neoplastic tissue, there are almost always normal, hyperplastic and/or dysplastic cells present in a sample. One must, therefore, be aware that genes obtained from a differential display experiment performed on an animal tissue model may not necessarily arise exclusively from the intended 'target' cells, e.g. hepatocytes/neoplastic cells. If appropriate, further analyses using immunohistochemistry, *in situ* hybridization or *in situ* RT-PCR should be used to confirm which cell types are expressing the gene(s) of interest. This problem is probably most acute for those studying the differential expression of genes in the development of different cell types, where there is a need to examine homologous cell populations. The problem is now being addressed at the National Cancer Institute (Bethesda, MD, USA) where new microdissection techniques have been employed to assist in their gene analysis programme, the Cancer Genome Anatomy Project (CGAP) (For more information see web site: <http://www.ncbi.nlm.nih.gov/ncicgap/intro.html>). There are also separation techniques available that utilise cell-specific antigens as a means to isolate target cells,

e.g. fluorescence activated cell sorting (FACS) (Dunbar *et al.* 1998, Kas-Deelen *et al.* 1998) and magnetic bead technology (Richard *et al.* 1998, Rogler *et al.* 1998).

However, those taking a holistic approach may consider this issue unimportant. There is an equally appropriate view that all those genes showing altered expression within a compromised tissue should be taken into consideration. After all, since all tissues are complex mixes of different, interacting cell types which intimately regulate each other's growth and development, it is clear that each cell type could in some way contribute (positively or negatively) towards the molecular mechanisms which lie behind responses to external stimuli or neoplastic growth. It is perhaps then more informative to carry out differential display experiments using *in vivo* as opposed to *in vitro* models, where uniform populations of identical cells probably represent a partial, skewed or even inaccurate picture of the molecular changes that occur.

The incidence and possible implications of inter-individual biological variation should be considered in any approach where whole animal models are being used. It is clear that individuals (humans and animals) respond in different ways to identical stimuli. One of the best characterized examples is the debrisoquine oxidation polymorphism, which is mediated by cytochrome CYP2D6 and determines the pharmacokinetics of many commonly prescribed drugs (Lennard 1993, Meyer and Zanger 1997). The reasons for such differences are varied and complex, but allelic variations, regulatory region polymorphisms and even physical and mental health can all contribute to observed differences in individual responses. Careful thought should, therefore, be given to the specific objectives of the study and to the possible value of pooling starting material (tissue/mRNA). The effect of this can be beneficial through the ironing out of exaggerated responses and unimportant minor fluctuations of (mechanistically) irrelevant genes in individual animals, thus providing a clearer overall picture of the general molecular mechanisms of the response. However, at the same time such minor variations may be of utmost importance in deciding the ability of individual animals to succumb to or resist the effects of a given chemical/disease.

How efficient are differential expression techniques at recovering a high percentage of differentially expressed genes?

A number of groups have produced experimental data suggesting that mammalian cells produce between 8000–15 000 different mRNA species at any one time (Mechler and Rabbitts 1981, Hedrick *et al.* 1984, Bravo 1990), although figures as high as 20–30 000 have also been quoted (Axel *et al.* 1976). Hedrick *et al.* (1984) provided evidence suggesting that the majority of these belong to the rare abundance class. A breakdown of this abundance distribution is shown in table 1.

When the results of differential display experiments have been compared with data obtained previously using other methods, it is apparent that not all differentially expressed mRNAs are represented in the final display. In particular, rare messages (which, importantly, often include regulatory proteins) are not easily recovered using differential display systems. This is a major shortcoming, as the majority of mRNA species exist at levels of less than 0.005% of the total population (table 1). Bertoli *et al.* (1995) examined the efficiency of DD templates (heterogeneous mRNA populations) for recovering rare messages and were unable to detect mRNA

species present at less than 1.2% of the total mRNA population—equivalent to an intermediate or abundant species. Interestingly, when simple model systems (single target only) were used instead of a heterogeneous mRNA population, the same primers could detect levels of target mRNA down to 10000× smaller. These results are probably best explained by competition for substrates from the many PCR products produced in a DD reaction.

The numbers of differentially expressed mRNAs reported in the literature using various model systems provides further evidence that many differentially expressed mRNAs are not recovered. For example, DeRisi *et al.* (1997) used DNA array technology to examine gene expression in yeast following exhaustion of sugar in the medium, and found that more than 1700 genes showed a change in expression of at least 2-fold. In light of such a finding, it would not be unreasonable to suggest that of the 8000–15 000 different mRNA species produced by any given mammalian cell, up to 1000 or more may show altered expression following chemical stimulation. Whilst this may be an extreme figure, it is known that at least 100 genes are activated/upregulated in Jurkat (T-) cells following IL-2 stimulation (Ullman *et al.* 1990). In addition, Wan *et al.* (1996) estimated that interferon- γ -stimulated HeLa cells differentially express up to 433 genes (assuming 24000 distinct mRNAs expressed by the cells). However, there have been few publications documenting anywhere near the recovery of these numbers. For example, in using DD to compare normal and regenerating mouse liver, Bauer *et al.* (1993) found only 70 of 38000 total bands to be different. Of these, 50% (35 genes) were shown to correspond to differentially expressed bands. Chen *et al.* (1996) reported 10 genes upregulated in female rat liver following ethinyl estradiol treatment. McKenzie and Drake (1997) identified 14 different gene products whose expression was altered by phorbol myristate acetate (PMA, a tumour promoter agent) stimulation of a human myelomonocytic cell line. Kilty and Vickers (1997) identified 10 different gene products whose expression was upregulated in the peripheral blood leukocytes of allergic disease sufferers. Linskens *et al.* (1995) found 23 genes differentially expressed between young and senescent fibroblasts. Techniques other than DD have also provided an apparent paucity of differentially expressed genes. Using SH for example, Cao *et al.* (1997) found 15 genes differentially expressed in colorectal cancer compared to normal mucosal epithelium. Fitzpatrick *et al.* (1995) isolated 17 genes upregulated in rat liver following treatment with the peroxisome proliferator, clofibrate; Philips *et al.* (1990) isolated 12 cDNA clones which were upregulated in highly metastatic mammary adenocarcinoma cell lines compared to poorly metastatic ones. Prashar and Weissman (1996) used 3' restriction fragment analysis and identified approximately 40 genes showing altered expression within 4 h of activation of Jurkat T-cells. Groenink and Leegwater (1996) analysed 27 gene fragments isolated using SSH of delayed early response phase of liver regeneration and found only 12 to be upregulated.

In the laboratory, SSH was used to isolate up to 70 candidate genes which appear to show altered expression in guinea pig liver following short-term treatment with the peroxisome proliferator, WY-14,643 (Rockett, Swales, Esdaile and Gibson, unpublished observations). However, these findings have still to be confirmed by analysis of the extracted tissue mRNA for differential expression of these sequences.

Whilst the latest differential display technologies are purported to include design and experimental modifications to overcome this lack of efficiency (in both the total number of differentially expressed genes recovered and the percentage that are true

positives), it is still not clear if such adaptations are practically effective—proving efficiency by spiking with a known amount of limited numbers of artificial construct(s) is one thing, but isolating a high percentage of the rare messages already present in an mRNA population is another. Of course, some models will genuinely produce only a small number of differentially expressed genes. In addition, there are also technical problems that can reduce efficiency. For example, mRNAs may have an unusual primary structure that effectively prevents their amplification by PCR-based systems. In addition, it is known that under certain circumstances not all mRNAs have 3' polyA sites. For example, during *Xenopus* development, deadenylation is used as a means to stabilize RNAs (Voeltz and Steitz 1998), whilst preferential deadenylation may play a role in regulating Hsp70 (and perhaps, therefore, other stress protein) expression in *Drosophila* (Dellavalle *et al.* 1994). The presence of deadenylated mRNAs would clearly reduce the efficiency of systems utilizing a polydT reverse transcription step. The efficiency of any system also depends on the quality of the starting material. All differential display techniques use mRNA as their target material. However, it is difficult to isolate mRNA that is completely free of ribosomal RNA. Even if polydT primers are used to prime first strand cDNA synthesis, ribosomal RNA is often transcribed to some degree (Clontech PCR-Select cDNA Subtraction kit user manual). It has been shown, at least in the case of SSH, that a high rRNA:mRNA ratio can lead to inefficient subtractive hybridization (Clontech PCR-Select cDNA Subtraction kit user manual), and there is no reason to suppose that it will not do likewise in other SH approaches. Finally, those techniques that utilise a presubtraction amplification step (e.g. RDA) may present a skewed representation since some sequences amplify better than others.

Of course, probably the most important consideration is the temporal factor. It is clear that any given differential display experiment can only interrogate a cell at one point in time. It may well be that a high percentage of the genes showing altered expression at that time are obtained. However, given that disease processes and responses to environmental stimuli involve dynamic cascades of signalling, regulation, production and action, it is clear that all those genes which are switched on/off at different times will not be recovered and, therefore, vital information may well be missed. It is, therefore, imperative to obtain as much information about the model system beforehand as possible, from which a strategy can be derived for targeting specific time points or events that are of particular interest to the investigator. One way of getting round this problem of single time point analysis is to conduct the experiment over a suitable time course which, of course, adds substantially to the amount of work involved.

How sensitive are differential expression technologies?

There has been little published data that addresses the issue of how large the change in expression must be for it to permit isolation of the gene in question with the various differential expression technologies. Although the isolation of genes whose expression is changed as little as 1.5-fold has been reported using SSH (Groenink and Leegwater 1996), it appears that those demonstrating a change in excess of 5-fold are more likely to be picked up. Thus, there is a 'grey zone' in between where small changes could fade in and out of isolation between

experiments and animals. DD, on the other hand, is not subject to this grey zone since, unlike SH approaches, it does not amplify the difference in expression between two samples. Wan *et al.* (1996) reported that differences in expression of twofold or more are detectable using DD.

Resolution and visualization of differential expression products

It seems highly improbable with current technology that a gel system could be developed that is able to resolve all gene species showing altered expression in any given test system (be it SH- or DD-based). Polyacrylamide gel electrophoresis (PAGE) can resolve size differences down to 0.2% (Sambrook *et al.* 1989) and are used as standard in DD experiments. Even so, it is clear that a complex series of gene products such as those seen in a DD will contain unresolvable components. Thus, what appears to be one band in a gel may in fact turn out to be several. Indeed, it has been well documented (Mathieu-Daude *et al.* 1996, Smith *et al.* 1997) that a single band extracted from a DD often represents a composite of heterogeneous products, and the same has been found for SSH displays in this laboratory (Rockett *et al.* 1997). One possible solution was offered by Mathieu-Daude *et al.* (1996), who extracted and reamplified candidate bands from a DD display and used single strand conformation polymorphism (SSCP) analysis to confirm which components represented the truly differentially expressed product.

Many scientists often try to avoid the use of PAGE where possible because it is technically more demanding than agarose gel electrophoresis (AGE). Unfortunately, high resolution agarose gels such as Metaphor (FMC, Lichfield, UK) and AquaPor HR (National Diagnostics, Hesse, UK), whilst easier to prepare and manipulate than PAGE, can only separate DNA sequences which differ in size by around 1.5–2% (15–20 base pairs for a 1Kb fragment). Thus, SSH, RDA or other such products which differ in size by less than this amount are normally not resolvable. However, a simple technique does in fact exist for increasing the resolving power of AGE—the inclusion of HA-red (10-phenyl neutral red-PEG ligand) or HA-yellow (bisbenzamide-PEG ligand) (Hanse Analytik GmbH, Bremen, Germany) in a gel separates identical or closely sized products on base content. Specifically, HA-red and -yellow selectively bind to GC and AT DNA motifs, respectively (Wawer *et al.* 1995, Hanse Analytik 1997, personal communication). Since both HA-stains possess an overall positive charge, they migrate towards the cathode when an electric field is applied. This is in direct opposition to DNA, which is negatively charged and, therefore, migrates towards the anode. Thus, if two DNA clones are identical in size (as perceived on a standard high resolution agarose gel), but differ in AT/GC content, inclusion of a HA-dye in the gel will effectively retard the migration of one of the sequences compared to the other, effectively making it apparently larger and, thus, providing a means of differentiating between the two. The use of HA-red has been shown to resolve sequences with an AT variation of less than 1% (Wawer *et al.* 1995), whilst Hanse Analytik have reported that HA staining is so sensitive that in one case it was used to distinguish two 567bp sequences which differed by only a single point mutation (Hanse Analytik 1996, personal communication). Therefore, if one wishes to check whether all the clones produced from a specific band in a differential display experiment are derived from the same gene species, a small amount of reamplified or digested clone can be run on a standard high resolution gel, and a second aliquot

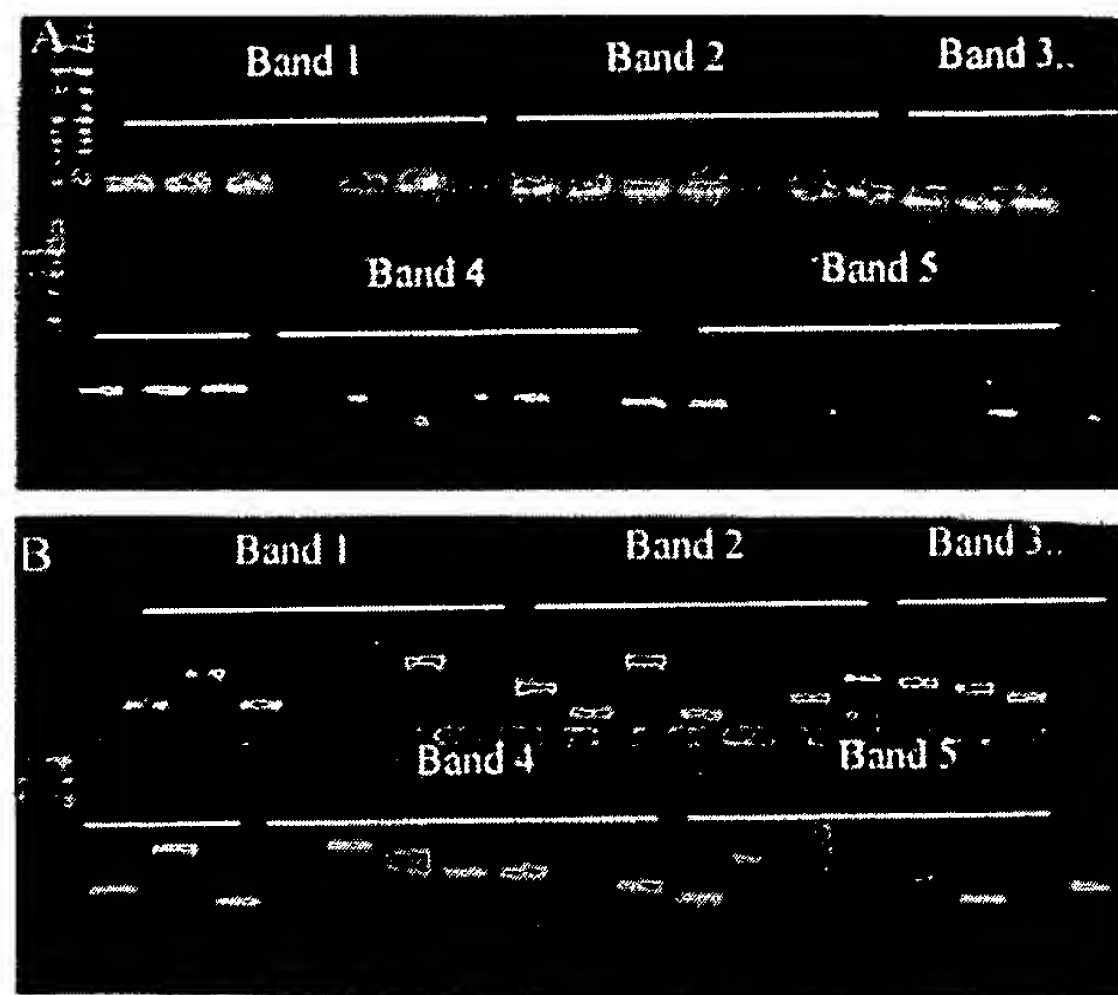


Figure 10. Discrimination of clones of identical/nearly identical size using HA-red. Bands of decreasing size (1–5) were extracted from the final display of a suppression subtractive hybridization experiment and cloned. Seven colonies were picked at random from each cloned band and their inserts amplified using PCR. The products were run on two gels, (A) a high resolution 2% agarose gel, and (B) a high resolution 2% agarose gel containing 1 U/ml HA-red. With few exceptions, all the clones from each band appear to be the same size (gel A). However, the presence of HA-red (gel B), which separates identically-sized DNA fragments based on the percentage of GC within the sequence, clearly indicates the presence of different gene species within each band. For example, even though all five re-amplified clones of band 1 appear to be the same size, at least four different gene species are represented.

in a similar gel containing one of the HA-stains. The standard gel should indicate any gross size differences, whilst the HA-stained gel should separate otherwise unresolvable species (on standard AGE) according to their base content. Geisinger *et al.* (1997) reported successful use of this approach for identifying DD-derived clones. Figure 10 shows such an experiment carried out in this laboratory on clones obtained from a band extracted from an SSH display.

An alternative approach is to carry out a 2-D analysis of the differential display products. In this approach, size-based separation is first carried out in a standard agarose gel. The gel slice containing the display is then extracted and incorporated in to a HA gel for resolution based on AT/GC content.

Of course, one should always consider the possibility of there being different gene species which are the same size and have the same GC/AT content. However, even these species are not unresolvable given some effort—again, one might use SSCP, or perhaps a denaturing gradient gel electrophoresis (DGGE) or temperature gradient field electrophoresis (TGGE) approach to resolve the contents of a band, either directly on the extracted band (Suzuki *et al.* 1991) or on the reamplified product.

The requirement of some differential display techniques to visualize large numbers of products (e.g. DD and GEF) can also present a problem in that, in terms of numbers, the resolution of PAGE rarely exceeds 300–400 bands. One approach to overcoming this might be to use 2-D gels such as those described by Uitterlinden *et al.* (1989) and Hatada *et al.* (1991).

Extraction of differentially expressed bands from a gel can be complex since, in some cases (e.g. DD, GEF), the results are visualized by autoradiographic means, such that precise overlay of the developed film on the gel must occur if the correct band is to be extracted for further analysis. Clearly, a misjudged extraction can account for many man-hours lost. This problem, and that of the use of radioisotopes, has been addressed by several groups. For example, Lohmann *et al.* (1995) demonstrated that silver staining can be used directly to visualize DD bands in horizontal PAGs. An *et al.* (1996) avoided the use of radioisotopes by transferring a small amount (20–30%) of the DNA from their DD to a nylon membrane, and visualizing the bands using chemiluminescent staining before going back to extract the remaining DNA from the gel. Chen and Peck (1996) went one step further and transferred the entire DD to a nylon membrane. The DNA bands were then visualized using a digoxigenin (DIG) system (DIG was attached to the polydT primers used in the differential display procedure). Differentially expressed bands were cut from the membrane and the DNA eluted by washing with PCR buffer prior to reamplification.

One of the advantages of using techniques such as SSH and RDA is that the final display can be run on an agarose gel and the bands visualized with simple ethidium bromide staining. Whilst this approach can provide acceptable results, overstaining with SYBR Green I or SYBR Gold nucleic acid stains (FMC) effectively enhances the intensity and sharpness of the bands. This greatly aids in their precise extraction and often reveals some faint products that may otherwise be overlooked. Whilst differential displays stained with SYBR Green I are better visualized using short wavelength UV (254 nm) rather than medium wavelength (306 nm), the shorter wavelength is much more DNA damaging. In practice, it takes only a few seconds to damage DNA extracted under 254 nm irradiation, effectively preventing reamplification and cloning. The best approach is to over stain with SYBR Green I and extract bands under a medium wavelength UV transillumination.

The possible use of 'microfingerprinting' to reduce complexity

Given the sheer number of gene products and the possible complexity of each band, an alternative approach to rapid characterization may be to use an enhanced analysis of a small section of a differential display—a 'sub-fingerprint' or 'micro-fingerprint'. In this case, one could concentrate on those bands which only appear in a particular chosen size region. Reducing the fingerprint in this way has at least two advantages. One is that it should be possible to use different gel types, concentrations and run times tailored exactly to that region. Currently, one might run products from 100–3000 + bp on the same gel, which leads to compromise in the gel system being used and consequently to suboptimal resolution, both in terms of size and numbers, and can lead to problems in the accurate excision of individual bands. Secondly, it may be possible to enhance resolution by using a 2-D analysis using a HA-stain, as described earlier. In summary, if a range of gene product sizes is carefully chosen to include certain 'relevant' genes, the 2-D system standardized, and appropriate gene analysis used, it may be possible to develop a method for the early and rapid identification of compounds which have similar or widely different cellular effects. If the prognosis for exposure to one or more other chemicals which display a similar profile is already known, then one could perhaps predict similar effects for any new compounds which show a similar micro-fingerprint.

An alternative approach to microfingerprinting is to examine altered expression in specific families of genes through careful selection of PCR primers and/or post-reaction analysis. Stress genes, growth factors and/or their receptors, cell cycling genes, cytochromes P450 and regulatory proteins might be considered as candidates for analysis in this way. Indeed, some off-the-shelf DNA arrays (e.g. Clontech's Atlas cDNA Expression Array series) already anticipated this to some degree by grouping together genes involved in different responses e.g. apoptosis, stress, DNA-damage response etc.

Screening

False positives

The generation of false positives has been discussed at length amongst the differential display community (Liang *et al.* 1993, 1995, Nishio *et al.* 1994, Sun *et al.* 1994, Sompayrac *et al.* 1995). The reason for false positives varies with the technique being used. For instance, in RDA, the use of adaptors which have not been HPLC purified can lead to the production of false positives through illegitimate ligation events (O'Neill and Sinclair 1997), whilst in DD they can arise through PCR artifacts and illegitimate transcription of rRNA. In SH, false positives appear to be derived largely from abundant gene species, although some may arise from cDNA/mRNA species which do not undergo hybridization for technical reasons.

A quick screening of putative differentially expressed clones can be carried out using a simple dot blot approach, in which labelled first strand probes synthesized from tester and driver mRNA are hybridized to an array of said clones (Hedrick *et al.* 1984, Sakaguchi *et al.* 1986). Differentially expressed clones will hybridize to tester probe, but not driver. The disadvantage of this approach is that rare species may not generate detectable hybridization signals. One option for those using SSH is to screen the clones using a labelled probe generated from the subtracted cDNA from which it was derived, and with a probe made from the reverse subtraction reaction (ClonTechniques 1997a). Since the SSH method enriches rare sequences, it should be possible to confirm the presence of clones representing low abundance genes. Despite this quick screening step, there is still the need to go back to the original mRNA and confirm the altered expression using a more quantitative approach. Although this may be achieved using Northern blots, the sensitivity is poor by today's high standards and one must rely on PCR methods for accurate and sensitive determinations (see below).

Sequence analysis

The majority of differential display procedures produce final products which are between 100 and 1000bp in size. However, this may considerably reduce the size of the sequence for analysis of the DNA databases. This in turn leads to a reduced confidence in the result—several families of genes have members whose DNA sequences are almost identical except in a few key stretches, e.g. the cytochrome P450 gene superfamily (Nelson *et al.* 1996). Thus, does the clone identified as being almost identical to gene X_0 really come from that gene, or its brother gene X_1 or its as yet undiscovered sister X_2 ? For example, using SSH, part of a gene was isolated,

which was up-regulated in the liver of rats exposed to Wy-14,643 and was identified by a FASTA search as being transferrin (data not shown). However, transferrin is known to be downregulated by hypolipidemic peroxisome proliferators such as Wy-14,643 (Hertz *et al.* 1996), and this was confirmed with subsequent RT-PCR analysis. This suggests that the gene sequence isolated may belong to a gene which is closely related to transferrin, but is regulated by a different mechanism.

A further problem associated with SH technology is redundancy. In most cases before SH is carried out, the cDNA population must first be simplified by restriction digestion. This is important for at least two reasons:

- (1) To reduce complexity—long cDNA fragments may form complex networks which prevent the formation of appropriate hybrids, especially at the high concentrations required for efficient hybridization.
- (2) Cutting the cDNAs into small fragments provides better representation of individual genes. This is because genes derived from related but distinct members of gene families often have similar coding sequences that may cross-hybridize and be eliminated during the subtraction procedure (Ko 1990). Furthermore, different fragments from the same cDNA may differ considerably in terms of hybridization and amplification and, thus, may not efficiently do one or the other (Wang and Brown 1991). Thus, some fragments from differentially expressed cDNAs may be eliminated during subtractive hybridization procedures. However, other fragments may be enriched and isolated. As a consequence of this, some genes will be cut one or more times, giving rise to two or more fragments of different sizes. If those same genes are differentially expressed, then two or more of the different size fragments may come through as separate bands on the final differential display, increasing the observed redundancy and increasing the number of redundant sequencing reactions.

Sequence comparisons also throw up another important point—at what degree of sequence similarity does one accept a result. Is 90% identity between a gene derived from your model species and another acceptably close? Is 95% between your sequence and one from the same species also acceptable? This problem is particularly relevant when the forward and reverse sequence comparisons give similar sequences with completely different gene species! An arbitrary decision seems to be to allocate genes that are definite (95% and above similarity) and then group those between 60 and 95% as being related or possible homologues.

Quantitative analysis

At some point, one must give consideration to the quantitative analysis of the candidate genes, either as a means of confirming that they are truly differentially expressed, or in order to establish just what the differences are. Northern blot analysis is a popular approach as it is relatively easy and quick to perform. However, the major drawback with Northern blots is that they are often not sensitive enough to detect rare sequences. Since the majority of messages expressed in a cell are of low abundance (see table 1), this is a major problem. Consequently, RT-PCR may be the method of choice for confirming differential expression. Although the procedure is somewhat more complex than Northern analysis, requiring synthesis of primers and optimization of reaction conditions for each gene species, it is now possible to set up high throughput PCR systems using multichannel pipettes, 96 +-well plates and

appropriate thermal cycling technology. Whilst quantitative analysis is more desirable, being more accurate and without reliance on an internal standard, the money and time needed to develop a competitor molecule is often excessive, especially when one might be examining tens or even hundreds of gene species. The use of semi-quantitative analysis is simpler, although still relatively involved. One must first of all choose an internal standard that does not change in the test cells compared to the controls. Numerous reference genes have been tried in the past, for example interferon-gamma (IFN- γ , Frye *et al.* 1989), β -actin (Heuval *et al.* 1994), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Wong *et al.* 1994), dihydrofolate reductase (DHFR, Mohler and Butler 1991), β -2-microglobulin (β -2-m, Murphy *et al.* 1990), hypoxanthine phosphoribosyl transferase (HPRT, Foss *et al.* 1998) and a number of others (ClonTechniques 1997b). Ideally, an internal standard should not change its level of expression in the cell regardless of cell age, stage in the cell cycle or through the effects of external stimuli. However, it has been shown on numerous occasions that the levels of most housekeeping genes currently used by the research community do in fact change under certain conditions and in different tissues (ClonTechniques 1997b). It is imperative, therefore, that preliminary experiments be carried out on a panel of housekeeping genes to establish their suitability for use in the model system.

Interpretation of quantitative data must also be treated with caution. By comparing the lists of genes identified by differential expression one can perhaps gain insight into why two different species react in different ways to external stimuli. For example, rats and mice appear sensitive to the non-genotoxic effects of a wide range of peroxisome proliferators whilst Syrian hamsters and guinea pigs are largely resistant (Orton *et al.* 1984, Rodricks and Turnbull 1987, Lake *et al.* 1989, 1993, Makowska *et al.* 1992). A simplified approach to resolving the reason(s) why is to compare lists of up- and down-regulated genes in order to identify those which are expressed in only one species and, through background knowledge of the effects of the said gene, might suggest a mechanism of facilitated non-genotoxic carcinogenesis or protection. Of course, the situation is likely to be far more complex. Perhaps if there were one key gene protecting guinea pig from non-genotoxic effects and it was upregulated 50 times by PPs, the same gene might only be up-regulated five times in the rat. However, since both were noted to be upregulated, the importance of the gene may be overlooked. Just to complicate matters, a large change in expression does not necessarily mean a biologically important change. For example, what is the true relevance of gene Y which shows a 50-fold increase after a particular treatment, and gene Z which shows only a 5-fold increase? If one examines the literature one may find that historically, gene Y has often been shown to be up-regulated 40–60-fold by a number of unrelated stimuli—in light of this the 50-fold increase would appear less significant. However, the literature may show that gene Z has never been recorded as having more than doubled in expression—which makes your 5-fold increase all the more exciting. Perhaps even more interesting is if that same 5-fold increase has only been seen in related neoplasms or following treatment with related chemicals.

Problems in using the differential display approach

Differential display technology originally held promise of an easily obtainable 'fingerprint' of those genes which are up- or down-regulated in test animals/cells in a developmental process or following exposure to given stimuli. However, it has

become clear that the fingerprinting process, whilst still valid, is much too complex to be represented by a single technique profile. This is because all differential display techniques have common and/or unique technical problems which preclude the isolation and identification of all those genes which show changes in expression. Furthermore, there are important genetic changes related to disease development which differential expression analysis is simply not designed to address. An example of this is the presence of small deletions, insertions, or point mutations such as those seen in activated oncogenes, tumour suppressor genes and individual polymorphisms. Polymorphic variations, small though they usually are, are often regarded as being of paramount importance in explaining why some patients respond better than others to certain drug treatments (and, in logical extension, why some people are less affected by potentially dangerous xenobiotics/carcinogens than others). The identification of such point mutations and naturally occurring polymorphisms requires the subsequent application of sequencing, SSCP, DGGE or TGGE to the gene of interest. Furthermore, differential display is not designed to address issues such as alternatively spliced gene species or whether an increased abundance of mRNA is a result of increased transcription or increased mRNA stability.

Conclusions

Perhaps the main advantage of open system differential display techniques is that they are not limited by extant theories or researcher bias in revealing genes which are differentially expressed, since they are designed to amplify all genes which demonstrate altered expression. This means that they are useful for the isolation of previously unknown genes which may turn out be useful biomarkers of a particular state or condition. At least one open system (SAGE) is also quantitative, thus eliminating the need to return to the original mRNA and carry out Northern/PCR analysis to confirm the result. However, the rapid progress of genome mapping projects means that over the next 5–10 years or so, the balance of experimental use will switch from open to closed differential display systems, particularly DNA arrays. Arrays are easier and faster to prepare and use, provide quantitative data, are suitable for high throughput analysis and can be tailored to look at specific signalling pathways or families of genes. Identification of all the gene sequences in human and common laboratory animals combined with improved DNA array technology, means that it will soon no longer be necessary to try to isolate differentially expressed genes using the technically more demanding open system approach. Thus, their main advantage (that of identifying unknown genes) will be largely eradicated. It is likely, therefore, that their sphere of application will be reduced to analysis of the less common laboratory species, since it will be some time yet before the genomes of such animals as zebrafish, electric eels, gerbils, crayfish and squid, for example, will be sequenced.

Of course, in the end the question will always remain: What is the functional/biological significance of the identified, differentially expressed genes? One persistent problem is understanding whether differentially expressed genes are a cause or consequence of the altered state. Furthermore, many chemicals, such as non-genotoxic carcinogens, are also mitogens and so genes associated with replication will also be upregulated but may have little or nothing to do with the

carcinogenic effect. Whilst differential display technology cannot hope to answer these questions, it does provide a springboard from which identification, regulatory and functional studies can be launched. Understanding the molecular mechanism of cellular responses is almost impossible without knowing the regulation and function of those genes and their condition (e.g. mutated). In an abstract sense, differential display can be likened to a still photograph, showing details of a fixed moment in time. Consider the Historian who knows the outcome of a battle and the placement and condition of the troops before the battle commenced, but is asked to try and deduce how the battle progressed and why it ended as it did from a few still photographs—an impossible task. In order to understand the battle, the Historian must find out the capabilities and motivation of the soldiers and their commanding officers, what the orders were and whether they were obeyed. He must examine the terrain, the remains of the battle and consider the effects the prevailing weather conditions exerted. Likewise, if mechanistic answers are to be forthcoming, the scientist must use differential display in combination with other techniques, such as knockout technology, the analysis of cell signalling pathways, mutation analysis and time and dose response analyses. Although this review has emphasized the importance of differential gene profiling, it should not be considered in isolation and the full impact of this approach will be strengthened if used in combination with functional genomics and proteomics (2-dimensional protein gels from isoelectric focusing and subsequent SDS electrophoresis and virtual 2D-maps using capillary electrophoresis). Proteomics is attracting much recent attention as many of the changes resulting in differential gene expression do not involve changes in mRNA levels, as described extensively herein, but rather protein-protein, protein-DNA and protein phosphorylation events which would require functional genomics or proteomic technologies for investigation.

Despite the limitations of differential display technology, it is clear that many potential applications and benefits can be obtained from characterizing the genetic changes that occur in a cell during normal and disease development and in response to chemical or biological insult. In light of functional data, such profiling will provide a 'fingerprint' of each stage of development or response, and in the long term should help in the elucidation of specific and sensitive biomarkers for different types of chemical/biological exposure and disease states. The potential medical and therapeutic benefits of understanding such molecular changes are almost immeasurable. Amongst other things, such fingerprints could indicate the family or even specific type of chemical an individual has been exposed to plus the length and/or acuteness of that exposure, thus indicating the most prudent treatment. They may also help uncover differences in histologically identical cancers, provide diagnostic tests for the earliest stages of neoplasia and, again, perhaps indicate the most efficacious treatment.

The Human Genome Project will be completed early in the next century and the DNA sequence of all the human genes will be known. The continuing development and evolution of differential gene expression technology will ensure that this knowledge contributes fully to the understanding of human disease processes.

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References

- ADAMS, M. D., KELLEY, J. M., GOCAYNE, J. D., DUBNICK, M., POLYMERPOULOS, M. H., XIAO, H., MERRIL, C. R., WU, A., OLDE, B., MORENO, R. F., KERLAVAGE, A. R., McCOMBIE, W. R. and VENTOR, J. C., 1991, Complementary DNA sequencing: expressed sequence tags and human genome project. *Science*, **252**, 1651–1656.
- AN, G., LUO, G., VELTRI, R. W. and O'HARA, S. M., 1996, Sensitive non-radioactive differential display method using chemiluminescent detection. *Biotechniques*, **20**, 342–346.
- AXEL, R., FEIGELSON, P. and SCHULTZ, G., 1976, Analysis of the complexity and diversity of mRNA from chicken liver and oviduct. *Cell*, **7**, 247–254.
- BAND, V. and SAGER, R., 1989, Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proceedings of the National Academy of Sciences, USA*, **86**, 1249–1253.
- BAUER, D., MULLER, H., REICH, J., RIEDEL, H., AHRENKIEL, V., WARTHOF, P. and STRAUSS, M., 1993, Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Research*, **21**, 4272–4280.
- BERTIOLI, D. J., SCHLICHTER, U. H. A., ADAMS, M. J., BURROWS, P. R., STEINBISS, H.-H. and ANTONIW, J. F., 1995, An analysis of differential display shows a strong bias towards high copy number mRNAs. *Nucleic Acids Research*, **23**, 4520–4523.
- BRAVO, R., 1990, Genes induced during the G0/G1 transition in mouse fibroblasts. *Seminars in Cancer Biology*, **1**, 37–46.
- BURN, T. C., PETROVICK, M. S., HOHAUS, S., ROLLINS, B. J. and TENEN, D. G., 1994, Monocyte chemoattractant protein-1 gene is expressed in activated neutrophils and retinoic acid-induced human myeloid cell lines. *Blood*, **84**, 2776–2783.
- CAO, J., CAI, X., ZHENG, L., GENG, L., SHI, Z., PAO, C. C. and ZHENG, S., 1997, Characterisation of colorectal cancer-related cDNA clones obtained by subtractive hybridisation screening. *Journal of Cancer Research and Clinical Oncology*, **123**, 447–451.
- CASSIDY, S. B., 1995, Uniparental disomy and genomic imprinting as causes of human genetic disease. *Environmental and Molecular Mutagenesis*, **25** (Suppl 26), 13–20.
- CHANG, G. W. and TERZAGHI-HOWE, M., 1998, Multiple changes in gene expression are associated with normal cell-induced modulation of the neoplastic phenotype. *Cancer Research*, **58**, 4445–4452.
- CHEN, J., SCHWARTZ, D. A., YOUNG, T. A., NORRIS, J. S. and YAGER, J. D., 1996, Identification of genes whose expression is altered during mitosuppression in livers of ethinyl estradiol-treated female rats. *Carcinogenesis*, **17**, 2783–2786.
- CHEN, J. J. W. and PECK, K., 1996, Non-radioactive differential display method to directly visualise and amplify differential bands on nylon membrane. *Nucleic Acid Research*, **24**, 793–794.
- CLON TECHNIQUES, 1997a, PCR-Select Differential Screening Kit—the nextstep after Clontech PCR-Select cDNA subtraction. *ClonTechniques*, **XII**, 18–19.
- CLON TECHNIQUES, 1997b, Housekeeping RT-PCR amplimers and cDNA probes. *ClonTechniques*, **XII**, 15–16.
- DAVIS, M. M., COHEN, D. I., NIELSEN, E. A., STEINMETZ, M., PAUL, W. E. and HOOD, L., 1984, Cell-type-specific cDNA probes and the murine I region: the localization and orientation of Ad alpha. *Proceedings of the National Academy of Sciences (USA)*, **81**, 2194–2198.
- DELLAVALLE, R. P., PETERSON, R. and LINDQUIST, S., 1994, Preferential deadenylation of HSP70 mRNA plays a key role in regulating Hsp70 expression in *Drosophila melanogaster*. *Molecular and Cell Biology*, **14**, 3646–3659.
- DERISI, J. L., VASHWANATH, R. L. and BROWN, P., 1997, Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science*, **278**, 680–686.
- DIATCHENKO, L., LAU, Y.-F. C., CAMPBELL, A. P., CHENCHIK, A., MOQADAM, F., HUANG, B., LUKYANOV, K., GURSKAYA, N., SVERDLOV, E. D. and SIEBERT, P. D., 1996, Suppression subtractive hybridisation: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences (USA)*, **93**, 6025–6030.
- DOGRA, S. C., WHITELAW, M. L. and MAY, B. K., 1998, Transcriptional activation of cytochrome P450 genes by different classes of chemical inducers. *Clinical and Experimental Pharmacology and Physiology*, **25**, 1–9.
- DUGUID, J. R. and DINAUER, M. C., 1990, Library subtraction of *in vitro* cDNA libraries to identify differentially expressed genes in scrapie infection. *Nucleic Acids Research*, **18**, 2789–2792.
- DUNBAR, P. R., OGG, G. S., CHEN, J., RUST, N., VAN DER BRUGGEN, P. and CERUNDOLO, V., 1998, Direct isolation, phenotyping and cloning of low-frequency antigen-specific cytotoxic T lymphocytes from peripheral blood. *Current Biology*, **26**, 413–416.

- FITZPATRICK, D. R., GERMAIN-LEE, E. and VALLE, D., 1995, Isolation and characterisation of rat and human cDNAs encoding a novel putative peroxisomal enoyl-CoA hydratase. *Genomics*, **27**, 457-466.
- FOSS, D. L., BAARSCH, M. J. and MURTAUGH, M. P., 1998, Regulation of hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase and beta-actin mRNA expression in porcine immune cells and tissues. *Animal Biotechnology*, **9**, 67-78.
- FRYE, R. A., BENZ, C. C. and LIU, E., 1989, Detection of amplified oncogenes by differential polymerase chain reaction. *Oncogene*, **4**, 1153-1157.
- GEISINGER, A., RODRIGUEZ, R., ROMERO, V. and WETTSTEIN, R., 1997, A simple method for screening cDNAs arising from the cloning of RNA differential display bands. *Elsevier Trends Journals Technical Tips Online*, <http://tto.trends.com>, document T01110.
- GRESS, T. M., HOHEISEL, J. D., LENNON, G. G., ZEHETNER, G. and LEHRACH, H., 1992, Hybridisation fingerprinting of high density cDNA filter arrays with cDNA pools derived from whole tissues. *Mammalian Genome*, **3**, 609-619.
- GRIFFIN, G. and KRISHNA, S., 1998, Cytokines in infectious diseases. *Journal of the Royal College of Physicians, London*, **32**, 195-198.
- GROENINK, M. and LEEGWATER, A. C. J., 1996, Isolation of delayed early genes associated with liver regeneration using Clontech PCR-select subtraction technique. *Clontechniques*, **XI**, 23-24.
- GUIMARAES, M. J., BAZAN, J. F., ZLOTNIK, A., WILES, M. V., GRIMALDI, J. C., LEE, F. and McCLANAHAN, T., 1995b, A new approach to the study of haematopoietic development in the yolk sac and embryoid bodies. *Development*, **121**, 3335-3346.
- GUIMARAES, M. J., LEE, F., ZLOTNIK, A. and McCLANAHAN, T., 1995a, Differential display by PCR: novel findings and applications. *Nucleic Acids Research*, **23**, 1832-1833.
- GURSKAYA, N. G., DIATCHENKO, L., CHENCHIK, P. D., SIEBERT, P. D., KHASPEKOV, G. L., LUKYANOV, K. A., VAGNER, L. L., ERMOLAEVA, O. D., LUKYANOV, S. A. and SVERDLOV, E. D., 1996, Equalising cDNA subtraction based on selective suppression of polymerase chain reaction: Cloning of Jurkat cell transcripts induced by phytohemagglutinin and phorbol 12-Myristate 13-Acetate. *Analytical Biochemistry*, **240**, 90-97.
- HAMPSON, I. N. and HAMPSON, L., 1997, CCLS and DROP—subtractive cloning made easy. *Life Science News* (A publication of Amersham Life Science), **23**, 22-24.
- HAMPSON, I. N., HAMPSON, L. and DEXTER, T. M., 1996, Directional random oligonucleotide primed (DROP) global amplification of cDNA: its application to subtractive cDNA cloning. *Nucleic Acids Research*, **24**, 4832-4835.
- HAMPSON, I. N., POPE, L., COWLING, G. J. and DEXTER, T. M., 1992, Chemical cross linking subtraction (CCLS): a new method for the generation of subtractive hybridisation probes. *Nucleic Acids Research*, **20**, 2899.
- HARA, E., KATO, T., NAKADA, S., SEKIYA, S. and ODA, K., 1991, Subtractive cDNA cloning using oligo(dT)30-latex and PCR: isolation of cDNA clones specific to undifferentiated human embryonal carcinoma cells. *Nucleic Acids Research*, **19**, 7097-7104.
- HATADA, I., HAYASHIZAKE, Y., HIROTSUNE, S., KOMATSUBARA, H. and MUKAI, T., 1991, A genomic scanning method for higher organisms using restriction sites as landmarks. *Proceedings of the National Academy of Sciences (USA)*, **88**, 9523-9527.
- HECHT, N., 1998, Molecular mechanisms of male sperm cell differentiation. *Bioessays*, **20**, 555-561.
- HEDRICK, S., COHEN, D. I., NIELSEN, E. A. and DAVIS, M. E., 1984, Isolation of T cell-specific membrane-associated proteins. *Nature*, **308**, 149-153.
- HERTZ, R., SECKBACH, M., ZAKIN, M. M. and BAR-TANA, J., 1996, Transcriptional suppression of the transferrin gene by hypolipidemic peroxisome proliferators. *Journal of Biological Chemistry*, **271**, 218-224.
- HEUVAL, J. P. V., CLARK, G. C., KOHN, M. C., TRITSCHER, A. M., GREENLEE, W. F., LUCIER, G. W. and BELL, D. A., 1994, Dioxin-responsive genes: Examination of dose-response relationships using quantitative reverse transcriptase-polymerase chain reaction. *Cancer Research*, **54**, 62-68.
- HILLIER, L. D., LENNON, G., BECKER, M., BONALDO, M. F., CHIAPELLI, B., CHISSOE, S., DIETRICH, N., DuBUQUE, T., FAVELLO, A., GISH, W., HAWKINS, M., HULTMAN, M., KUCABA, T., LACY, M., LE, M., LE, N., MARDIS, E., MOORE, B., MORRIS, M., PARSONS, J., PRANGE, C., RIFKIN, L., ROHLFING, T., SCHELLENBERG, K., SOARES, M. B., TAN, F., THIERRY-MEG, J., TREVASKIS, E., UNDERWOOD, K., WOHLDMAN, P., WATERSTON, R., WILSON, R. and MARRA, M., 1996, Generation and analysis of 280,000 human expressed sequence tags. *Genome Research*, **6**, 807-828.
- HUBANK, M. and SCHATZ, D. G., 1994, Identifying differences in mRNA expression by representational difference analysis. *Nucleic Acids Research*, **22**, 5640-5648.
- HUNTER, T., 1991, Cooperation between oncogenes. *Cell*, **64**, 249-270.
- IVANOVA, N. B. and BELYAVSKY, A. V., 1995, Identification of differentially expressed genes by restriction endonuclease-based gene expression fingerprinting. *Nucleic Acids Research*, **23**, 2954-2958.
- JAMES, B. D. and HIGGINS, S. J., 1985, *Nucleic Acid Hybridisation* (Oxford: IRL Press Ltd).
- KAS-DEELEN, A. M., HARMSSEN, M. C., DE MAAR, E. F. and VAN SON, W. J., 1998, A sensitive method for

- quantifying cytomegalic endothelial cells in peripheral blood from cytomegalovirus-infected patients. *Clinical Diagnostic and Laboratory Immunology*, 5, 622-626.
- KILTY, I. and VICKERS, P., 1997, Fractionating DNA fragments generated by differential display PCR. *Strategies Newsletter* (Stratagene), 10, 50-51.
- KLEINJAN, D.-J. and VAN HEYNINGEN, V., 1998, Position effect in human genetic disease. *Human and Molecular Genetics*, 7, 1611-1618.
- KO, M. S., 1990, An 'equalized cDNA library' by the reassociation of short double-stranded cDNAs. *Nucleic Acids Research*, 18, 5705-5711.
- LAKE, B. G., EVANS, J. G., CUNNINGHAME, M. E. and PRICE, R. J., 1993, Comparison of the hepatic effects of Wy-14,643 on peroxisome proliferation and cell replication in the rat and Syrian hamster. *Environmental Health Perspectives*, 101, 241-248.
- LAKE, B. G., EVANS, J. G., GRAY, T. J. B., KOROSI, S. A. and NORTH, C. J., 1989, Comparative studies of nafenopin-induced hepatic peroxisome proliferation in the rat, Syrian hamster, guinea pig and marmoset. *Toxicology and Applied Pharmacology*, 99, 148-160.
- LENNARD, M. S., 1993, Genetically determined adverse drug reactions involving metabolism. *Drug Safety*, 9, 60-77.
- LEVY, S., TODD, S. C. and MAECKER, H. T., 1998, CD81(TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annual Review of Immunology*, 16, 89-109.
- LIANG, P. and PARDEE, A. B., 1992, Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, 257, 967-971.
- LIANG, P., AVERBOUKH, L., KEYOMARSI, K., SAGER, R. and PARDEE, A., 1992, Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelial cells. *Cancer Research*, 52, 6966-6968.
- LIANG, P., AVERBOUKH, L. and PARDEE, A. B., 1993, Distribution & cloning of eukaryotic mRNAs by means of differential display refinements and optimisation. *Nucleic Acids Research*, 21, 3269-3275.
- LIANG, P., BAUER, D., AVERBOUKH, L., WARTHOF, P., ROHRWILD, M., MULLER, H., STRAUSS, M. and PARDEE, A. B., 1995, Analysis of altered gene expression by differential display. *Methods in Enzymology*, 254, 304-321.
- LINSKENS, M. H., FENG, J., ANDREWS, W. H., ENLOW, B. E., SAATI, S. M., TONKIN, L. A., FUNK, W. D. and VILLEPONTEAU, B., 1995, Cataloging altered gene expression in young and senescent cells using enhanced differential display. *Nucleic Acids Research*, 23, 3244-3251.
- LISITSYN, N., LISITSYN, N. and WIGLER, M., 1993, Cloning the differences between two complex genomes. *Science*, 259, 946-951.
- LOHMANN, J., SCHICKLE, H. and BOSCH, T. C. G., 1995, REN Display, a rapid and efficient method for non-radioactive differential display and mRNA isolation. *Biotechniques*, 18, 200-202.
- LUNNEY, J. K., 1998, Cytokines orchestrating the immune response. *Reviews in Science and Technology*, 17, 84-94.
- MAKOWSKA, J. M., GIBSON, G. G. and BONNER, F. W., 1992, Species differences in ciprofibrate-induction of hepatic cytochrome P450A1 and peroxisome proliferation. *Journal of Biochemical Toxicology*, 7, 183-191.
- MALDARELLI, F., XIANG, C., CHAMOUN, G. and ZEICHNER, S. L., 1998, The expression of the essential nuclear splicing factor SC35 is altered by human immunodeficiency virus infection. *Virus Research*, 53, 39-51.
- MATHIEU-DAUDE, F., CHENG, R., WELSH, J. and MCCLELLAND, M., 1996, Screening of differentially amplified cDNA products from RNA arbitrarily primed PCR fingerprints using single strand conformation polymorphism (SSCP) gels. *Nucleic Acids Research*, 24, 1504-1507.
- MCKENZIE, D. and DRAKE, D., 1997, Identification of differentially expressed gene products with the castaway system. *Strategies Newsletter* (Stratagene), 10, 19-20.
- MCCLELLAND, M., MATHIEU-DAUDE, F. and WELSH, J., 1996, RNA fingerprinting and differential display using arbitrarily primed PCR. *Trends in Genetics*, 11, 242-246.
- MECHLER, B. and RABBITTS, T. H., 1981, Membrane-bound ribosomes of myeloma cells. IV. mRNA complexity of free and membrane-bound polysomes. *Journal of Cell Biology*, 88, 29-36.
- MEYER, U. A. and ZANGER, U. M., 1997, Molecular mechanisms of genetic polymorphisms of drug metabolism. *Annual Review of Pharmacology and Toxicology*, 37, 269-296.
- MOHLER, K. M. and BUTLER, L. D., 1991, Quantitation of cytokine mRNA levels utilizing the reverse transcriptase-polymerase chain reaction following primary antigen-specific sensitization in vivo—I. Verification of linearity, reproducibility and specificity. *Molecular Immunology*, 28, 437-447.
- MURPHY, L. D., HERZOG, C. E., RUDICK, J. B., TITO FOJO, A. and BATES, S. E., 1990, Use of the polymerase chain reaction in the quantitation of the *mdr-1* gene expression. *Biochemistry*, 29, 10351-10356.
- NELSON, D. R., KOYMANS, L., KAMATAKI, T., STEGEMAN, J. J., FEYEREISEN, R., WAXMAN, D. J., WATERMAN, M. R., GOTOH, O., COON, M. J., ESTABTROOK, R. W., GUNSALUS, I. C. and NEBERT, D. W., 1996, Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, 6, 1-42.

- NISHIO, Y., AIELLO, L. P. and KING, G. L., 1994, Glucose induced genes in bovine aortic smooth muscle cells identified by mRNA differential display. *FASEB Journal*, **8**, 103–106.
- O'NEILL, M. J. and SINCLAIR, A. H., 1997, Isolation of rare transcripts by representational difference analysis. *Nucleic Acids Research*, **25**, 2681–2682.
- ORTON, T. C., ADAM, H. K., BENTLEY, M., HOLLOWAY, B. and TUCKER, M. J., 1984, Clobazart: species differences in the morphological and biochemical response of the liver following chronic administration. *Toxicology and Applied Pharmacology*, **73**, 138–151.
- PELKONEN, O., MAENPAA, J., TAAVITSAINEN, P., RAUTIO, A. and RAUNIO, H., 1998, Inhibition and Induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica*, **28**, 1203–1253.
- PHILIPS, S. M., BENDALL, A. J. and RAMSHAW, I. A., 1990, Isolation of genes associated with high metastatic potential in rat mammary adenocarcinomas. *Journal of the National Cancer Institute*, **82**, 199–203.
- PRASHAR, Y. and WEISSMAN, S. M., 1996, Analysis of differential gene expression by display of 3' end restriction fragments of cDNAs. *Proceedings of the National Academy of Sciences (USA)*, **93**, 659–663.
- RAGNO, S., ESTRADA, I., BUTLER, R. and COLSTON, M. J., 1997, Regulation of macrophage gene expression following invasion by *Mycobacterium tuberculosis*. *Immunology Letters*, **57**, 143–146.
- RAMANA, K. V. and KOHLI, K. K., 1998, Gene regulation of cytochrome P450—an overview. *Indian Journal of Experimental Biology*, **36**, 437–446.
- RICHARD, L., VELASCO, P. and DETMAR, M., 1998, A simple immunomagnetic protocol for the selective isolation and long-term culture of human dermal microvascular endothelial cells. *Experimental Cell Research*, **240**, 1–6.
- ROCKETT, J. C., ESDAILE, D. J. and GIBSON, G. G., 1997, Molecular profiling of non-genotoxic hepatocarcinogenesis using differential display reverse transcription-polymerase chain reaction (ddRT-PCR). *European Journal of Drug Metabolism and Pharmacokinetics*, **22**, 329–333.
- RODRICKS, J. V. and TURNBULL, D., 1987, Inter-species differences in peroxisomes and peroxisome proliferation. *Toxicology and Industrial Health*, **3**, 197–212.
- ROGLER, G., HAUSMANN, M., VOGL, D., ASCHENBRENNER, E., ANDUS, T., FALK, W., ANDRESEN, R., SCHOLMERICH, J. and GROSS, V., 1998, Isolation and phenotypic characterization of colonic macrophages. *Clinical and Experimental Immunology*, **112**, 205–215.
- ROHN, W. M., LEE, Y. J. and BENVENISTE, E. N., 1996, Regulation of class II MHC expression. *Critical Reviews in Immunology*, **16**, 311–330.
- RUDIN, C. M. and THOMPSON, C. B., 1998, B-cell development and maturation. *Seminars in Oncology*, **25**, 435–446.
- SAKAGUCHI, N., BERGER, C. N. and MELCHERS, F., 1986, Isolation of a cDNA copy of an RNA species expressed in murine pre-B cells. *EMBO Journal*, **5**, 2139–2147.
- SAMBROOK, J., FRITSCH, E. F. and MANIATIS, T., 1989, Gel electrophoresis of DNA. In N. Ford, M. Nolan and M. Ferguson (eds), *Molecular Cloning—A laboratory manual*, 2nd edition (New York: Cold Spring Harbour Laboratory Press), Volume 1, pp. 6–37.
- SARGENT, T. D. and DAWID, I. B., 1983, Differential gene expression in the gastrula of *Xenopus laevis*. *Science*, **222**, 135–139.
- SCHENA, M., SHALON, D., HELLER, R., CHAI, A., BROWN, P. O. and DAVIS, R. W., 1996, Parallel human genome analysis: Microarray-based expression monitoring of 1000 genes. *Proceedings of the National Academy of Sciences (USA)*, **93**, 10614–10619.
- SCHNEIDER, C., KING, R. M. and PHILIPSON, L., 1988, Genes specifically expressed at growth arrest of mammalian cells. *Cell*, **54**, 787–793.
- SCHNEIDER-MAUNOURY, S., GILARDI-HEBENSTREIT, P. and CHARNAY, P., 1998, How to build a vertebrate hindbrain. Lessons from genetics. *C R Academy of Science III*, **321**, 819–834.
- SEMENZA, G. L., 1994, Transcriptional regulation of gene expression: mechanisms and pathophysiology. *Human Mutations*, **3**, 180–199.
- SEWALL, C. H., BELL, D. A., CLARK, G. C., TRITSCHER, A. M., TULLY, D. B., VANDEN HEUVEL, J. and LUCIER, G. W., 1995, Induced gene transcription: implications for biomarkers. *Clinical Chemistry*, **41**, 1829–1834.
- SINGH, N., AGRAWAL, S. and RASTOGI, A. K., 1997, Infectious diseases and immunity: special reference to major histocompatibility complex. *Emerging Infectious Diseases*, **3**, 41–49.
- SMITH, N. R., LI, A., ALDERSLEY, M., HIGH, A. S., MARKHAM, A. F. and ROBINSON, P. A., 1997, Rapid determination of the complexity of cDNA bands extracted from DDRT-PCR polyacrylamide gels. *Nucleic Acids Research*, **25**, 3552–3554.
- SOMPAYRAC, L., JANE, S., BURN, T. C., TENEN, D. G. and DANNA, K. J., 1995, Overcoming limitations of the mRNA differential display technique. *Nucleic Acids Research*, **23**, 4738–4739.
- ST JOHN, T. P. and DAVIS, R. W., 1979, Isolation of galactose-inducible DNA sequences from *Saccharomyces cerevisiae* by differential plaque filter hybridisation. *Cell*, **16**, 443–452.
- SUN, Y., HEGAMYER, G. and COLBURN, N. H., 1994, Molecular cloning of five messenger RNAs differentially expressed in preneoplastic or neoplastic JB6 mouse epidermal cells: one is homologous to human tissue inhibitor of metalloproteinases-3. *Cancer Research*, **54**, 1139–1144.

- SUNG, Y. J. and DENMAN, R. B., 1997, Use of two reverse transcriptases eliminates false-positive results in differential display. *Biotechniques*, **23**, 462–464.
- SUTTON, G., WHITE, O., ADAMS, M. and KERLAVAGE, A., 1995, TIGR Assembler; A new tool for assembling large shotgun sequencing projects. *Genome Science and Technology*, **1**, 9–19.
- SUZUKI, Y., SEKIYA, T. and HAYASHI, K., 1991, Allele-specific polymerase chain reaction: a method for amplification and sequence determination of a single component among a mixture of sequence variants. *Analytical Biochemistry*, **192**, 82–84.
- SYED, V., GU, W. and HECHT, N. B., 1997, Sertoli cells in culture and mRNA differential display provide a sensitive early warning assay system to detect changes induced by xenobiotics. *Journal of Andrology*, **18**, 264–273.
- UITTERLINDEN, A. G., SLAGBOOM, P., KNOOK, D. L. and VUGL, J., 1989, Two-dimensional DNA fingerprinting of human individuals. *Proceedings of the National Academy of Sciences (USA)*, **86**, 2742–2746.
- ULLMAN, K. S., NORTHROP, J. P., VERWEIJ, C. L. and CRABTREE, G. R., 1990, Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. *Annual Review of Immunology*, **8**, 421–452.
- VASMATZIS, G., ESSAND, M., BRINKMANN, U., LEE, B. and PASTON, I., 1998, Discovery of three genes specifically expressed in human prostate by expressed sequence tag database analysis. *Proceedings of the National Academy of Sciences (USA)*, **95**, 300–304.
- VELCULESCU, V. E., ZHANG, L., VOGELSTEIN, B. and KINZLER, K. W., 1995, Serial analysis of gene expression. *Science*, **270**, 484–487.
- VOELTZ, G. K. and STEITZ, J. A., 1998, AuuuA sequences direct mRNA deadenylation uncoupled from decay during *Xenopus* early development. *Molecular and Cell Biology*, **18**, 7537–7545.
- VOGELSTEIN, B. and KINZLER, K. W., 1993, The multistep nature of cancer. *Trends in Genetics*, **9**, 138–141.
- WALTER, J., BELFIELD, M., HAMPSON, I. and READ, C., 1997, A novel approach for generating subtractive probes for differential screening by CCLS. *Life Science News*, **21**, 13–14.
- WAN, J. S., SHARP, S. J., POIRIER, G. M.-C., WAGAMAN, P. C., CHAMBERS, J., PYATI, J., HOM, Y.-L., GALINDO, J. E., HUVAR, A., PETERSON, P. A., JACKSON, M. R. and ERLANDER, M. G., 1996, Cloning differentially expressed mRNAs. *Nature Biotechnology*, **14**, 1685–1691.
- WALTER, J., BELFIELD, M., HAMPSON, I. and READ, C., 1997, A novel approach for generating subtractive probes for differential screening by CCLS. *Life Science News*, **21**, 13–14.
- WANG, Z. and BROWN, D. D., 1991, A gene expression screen. *Proceedings of the National Academy of Sciences (USA)*, **88**, 11505–11509.
- WAWER, C., RUGGEBERG, H., MEYER, G. and MUYZER, G., 1995, A simple and rapid electrophoresis method to detect sequence variation in PCR-amplified DNA fragments. *Nucleic Acids Research*, **23**, 4928–4929.
- WELSH, J., CHADA, K., DALAL, S. S., CHENG, R., RALPH, D. and MCCLELLAND, M., 1992, Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Research*, **20**, 4965–4970.
- WONG, H., ANDERSON, W. D., CHENG, T. and RIABOWOL, K. T., 1994, Monitoring mRNA expression by polymerase chain reaction: the 'primer-dropping' method. *Analytical Biochemistry*, **223**, 251–258.
- WONG, K. K. and MCCLELLAND, M., 1994, Stress-inducible gene of *Salmonella typhimurium* identified by arbitrarily primed PCR of RNA. *Proceedings of the National Academy of Sciences (USA)*, **91**, 639–643.
- WYNFORD-THOMAS, D., 1991, Oncogenes and anti-oncogenes; the molecular basis of tumour behaviour. *Journal of Pathology*, **165**, 187–201.
- XHU, D., CHAN, W. L., LEUNG, B. P., HUANG, F. P., WHEELER, R., PIEDRAFITA, D., ROBINSON, J. H. and LIEW, F. Y., 1998, Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *Journal of Experimental Medicine*, **187**, 787–794.
- YANG, M. and SYTOWSKI, A. J., 1996, Cloning differentially expressed genes by linker capture subtraction. *Analytical Biochemistry*, **237**, 109–114.
- ZHAO, N., HASHIDA, H., TAKAHASHI, N., MISUMI, Y. and SAKAKI, Y., 1995, High-density cDNA filter analysis: a novel approach for large scale quantitative analysis of gene expression. *Gene*, **156**, 207–213.
- ZHAO, X. J., NEWSOME, J. T. and CIHLAR, R. L., 1998, Up-regulation of two *Candida albicans* genes in the rat model of oral candidiasis detected by differential display. *Microbial Pathogenesis*, **25**, 121–129.
- ZIMMERMANN, C. R., ORR, W. C., LECLERC, R. F., BARNARD, C. and TIMBERLAKE, W. E., 1980, Molecular cloning and selection of genes regulated in *Aspergillus* development. *Cell*, **21**, 709–715.